GENETIC ANALYSIS OF THE NITRATE REDUCTASE GENE OF *PROPIONIBACTERIUM*

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Chapter I

INTRODUCTION

Bacteria of the genus *Propionibacterium* are gram positive, non motile, non-sporeforming, pleomorphic rods (Cummins and Johnson, 1986). They are anaerobic to aerotolerant and are generally catalase positive. The "classical propionibacteria" or "dairy propionibacteria" are those naturally found in cheese and dairy products, although they have been found in other natural fermentations. These bacteria are presently being used in the dairy industry as starter cultures for swiss cheese production (Biede and Hammond 1979; Langsgrud and Reinbold, 1973a; Langsgrud and Reinbold 1973b), as silage and grain inoculants (Woolford, 1975; Flores-Galarza, et al., 1985; Raeker, et al., 1992; Dawson, et al., 1991; Tomes, 1991), and for production of propionic acid and vitamin B_{12} (Perlman 1978, Playne 1985). Propionibacteria have also been found to be useful as human probiotics (Sidorchuk and Bondarko, 1984; Sidorchuk, et al., 1984; Korneya, 1982; Nabukhotnyi, 1985; Mantere-Alhonen, 1987; Sanigullina, 1985; Cerna, 1984; Somkuti and Johnson, 1990) and direct-fed microbials for animals (Vladimirov, et al., 1978; Tuikov, 1982; Mantre-Althonen, 1982;1983;1985; Antipov, 1980).

Recently, research has examined the use of propionibacteria as a direct-fed microbial to reduce the toxic effects of nitrite in cattle (Rehberger, et al., 1993). Although a commercial application has been developed, nothing is currently known about the genetics of the denitrification process of *Propionibacterium*. Therefore this present study

was initiated in order to better understand the denitrification process of propionibacteria which may lead to the development of an improved direct-fed microbial.

The purpose of this investigation was to isolate and characterize the gene for the dissimilatory nitrate reductase enzyme in propionibacteria. The major components of this study include amplification of nitrate reductase conserved regions using the polymerase chain reaction, Southern hybridization for comparisons of the nitrate reductase among propionibacteria strains, and restriction endonuclease digestions and DNA sequencing analysis for comparisons to a conserved region in the nitrate reductase of *Escherichia coli*.

REVIEW OF LITERATURE

Genus of *Propionibacterium*

Bacteria of the genus *Propionibacterium* are gram positive, non-motile, non sporeforming, pleomorphic rods. They are anaerobic to aerotolerant and generally catalase positive (Cummins and Johnson, 1986). This phenomenon distinguishes propionibacteria from most anaerobes which are generally catalase negative. Growth of cells occurs most rapidly from 30-37 °C. They are often diptheroid or club-shaped, with one end rounded and the other tapered or pointed. However, cells may be coccoid, bifid or even branched. Cells may occur singly, in pairs, or short chains in X or Y configurations or clumping in "Chinese character" arrangements. Propionibacteria are chemorganotrophs that produce large amounts of propionic acid, acetic acid, and CO_2 as fermentation products. In lesser amounts these bacteria generally also produce iso-valeric, formic, succinic or lactic acids. Typical colonies may be white, gray, pink, red, yellow or orange in color. G+C contents of bacterial DNA range from 53-67% (Tm) (Cummins and Johnson, 1986).

Members of this genus are commonly divided into two groups, differing in their natural habitat, the first being those naturally found in cheese and dairy products which are appropriately named the "dairy propionibacteria" or "classical propionibacteria". These bacterial strains have also been found in other natural fermentations such as silage, fermenting olives and soil (Cummins and Johnson, 1986). Propionibacteria strains found primarily on the skin compose the "acnes group strains" or "cutaneous propionibacteria".

These bacteria have also been found to occur elsewhere other than the skin such as in the human intestine.

Presently propionibacteria have a number of useful applications. The bacteria's most common application is in the Dairy industry as starter cultures for the manufacture of Swiss-type cheeses. Propionibacteria ferment the lactic acid produced from lactic acid starter cultures. This fermentation process produces the characteristic eyes and plays a part in developing the typical flavor of Swiss cheese (Biede and Hammond 1979, Langsgrud and Reinbold 1973a, Langsgrud and Reinbold 1973b).
Unfortunately, propionibacteria have been implicated for a number of Swiss cheese defects (Hettinga and Reinbold, 1975; Hettinga et al.,1974; Langsrud and Reinbold, 1973; Park et al., 1967).

Propionibacteria have also been used as human probiotics. A number of successful experiments have produced a milk containing propionibacteria and *Lactobacillus acidophilus* and some were tested as prophylactic agents against gastrointestinal ailments (Sidorchuk and Bondarko, 1984; Sidorchuk et al., 1984, Korneya, 1982; Nabukhotnyi, 1985; Mantere-Alhonen, 1987; Sanigullina, 1985). Elvit is another milk product which is enriched with vitamin B_{12} and folacin by the addition of *Propionibacteria shermanii* (Cerna, 1984). This is expected to be favorable for humans especially for the formation of red corpuscles . In another study, *Propionibacteria freudenreichii* was found to have the ability to take up cholesterol in media broth, which may be an indication of action within the human organism (Somkuti and Johnson, 1990).

The use of propionibacteria as silage inoculant has been a subject of several studies. *Propionibacteria* spp. and *Micrococcus lactilyticus* were both isolated from silage and both demonstrated lactic acid fermentation abilities (Woolford, 1975). Propionibacteria in combination with *Lactobacillus plantarum* proved to be an effective means of preservation for high moisture corn (Flores-Galarza et al., 1985). By lowering the pH, all mold growth was prevented and a drastic reduction in initial yeast populations

were observed. In one study, fermentation broths from *P. theonii* were added to high-moisture corn samples as was pure propionic acid (Raeker et al., 1992). Both methods were equal in abilities as fungicidal agents, preventing growth of inoculated *Aspergillus flavus* and maintaining mold-free conditions for more than a year. *P. acidipropionici* and *P. freudenreichii* were used to inoculate reconstituted corn, resulting in increased propionic acid production and decreased yeasts and molds (Dawson et al., 1991). A patented method to preserve silage has been demonstrated using two strains of *Propionibacterium jensenii* (Tomes, 1991).

Several other industrial applications of propionibacteria have been described including the production of vitamin B_{12} and propionic acid (Perlman, 1978; Playne, 1985). Propionibacteria have been used in industry to produce vitamin B_{12}, although the development of *Pseudomonas* strains with increased yields of the vitamin may limit the future of this application (Crueger, 1982). However a number of recent studies have shown the capability of propionibacteria strains to produce vitamin B_{12} from waste products of lime processing and whey from other manufacturing processes (Marwaha et al.,1983; Perez-Mendoza and Herenandez,1983; Yongsmith, 1983). One patented process has produced mycostatic whey using *Propionibacteria acidipropionici* (Anderson, 1985).

To date, production of propionic acid from propionibacteria is not an economically competitive process, although improvements in processes are being continuously made (Brumm, 1989). Propionic acid is used widely to produce cellulose plastics, herbicides, and perfumes (Playne, 1985). Propionic acid is also used as a mold inhibitor to preserve feed and food products (Playne, 1985; Tabib, 1982).

Revealing studies about the production of bacterocins from propionibacteria are providing more information about the bacteria's future use as an antimicrobial agent. A very proteolytic sensitive and heat labile bacterocin was isolated from *Propionibacteria theonii*. This enzyme was inhibitory to a number of lactobacilli strains, gram negative pathogens, yeasts and molds (Lyon and Glatz, 1991). A heat stable bacterocin is

produced by *Propionibacterium jensenii*, however further research revealed that the enzyme only inhibited certain strains of lactobacilli, lactococci and propionibacteria. None of the pathogenic bacteria tested were inhibited by the enzyme (Grinstead and Barefoot, 1992). Recent work in our laboratory has identified a heat stable bacterocin from *Propionibacterium freudenreichii* that inhibits gram negative pathogenic and non-pathogenic strains of bacteria (Mao and Rehberger, unpublished).

One final application for the use of propionibacteria is as a direct fed microbial for animals. Propionibacteria, lactic acid bacteria, and on one occasion antibiotics have been used together in various combinations to increase weight gain in cattle (Vladimirov et al. 1978; Tuikov et al., 1982). Studies with various animal species and *in vitro* simulations involving propionibacteria concluded that the growth promoting properties of propionibacteria were caused in part by the production of propionic acid, vitamin B_{12} synthesis, and antioxidant action (Mantre-Althonen, 1982;1983;1985). One publication reviewed a number of studies using probiotics in the USSR and other countries (Antipov, 1980). It was emphasized that Propiovit, the propionic acid based bacteria product and the other similar probiotics prevent diseases by improving the gastrointestinal microflora but have little therapeutic effect.

Recent work in our laboratory has provided another facet to the application of propionibacteria as an animal direct-fed microbial. *Propionibacteria acidipropionici* (P5) a known denitrifier, was given as a direct-fed microbial to reduce the effects of nitrate toxicity (Rehberger et al., 1993). This study demonstrated the ability of *Propionibacteria acidipropionici* to reduce toxic ruminal nitrite levels, both *in vivo* and simulated *in vitro* experiments. Inoculation with propionibacteria reduced ruminal nitrite load by 40-46%. This study suggested that continuous inoculation of propionibacteria will have a significant prophylaxis value against nitrate toxicity due to feeding of high nitrate containing forage.

Strain improvements will open the door for even more applications which have yet to become economically proficient. A better understanding of the genetics of these organisms will be the first step in such improvements.

Nitrate-Nitrite Poisoning in Cattle

A number of factors dependent on the plant, environment, and management of forage can lead to elevated nitrate levels (Kilgore, 1993). The plant species, stage of growth and the part of the plant are factors which affect the levels of nitrate. Environmental conditions such as drought, and reduced sunlight, frost, hail and diseases which cause leaf damage also lead to nitrate accumulation. Low temperatures which slow down photosynthesis in warm season plants favor increasing nitrate levels. Management efforts for improved forage production including fertilization and herbicidal use along with the types of harvesting techniques used have major effects on the nitrate concentrations within forage.

Nitrate within the forage is reduced to nitrite and further to ammonia by microorganisms in the rumen of cattle. When high levels of nitrate are consumed, the formation of nitrite exceeds the rate of nitrite breakdown, resulting in elevated nitrite concentration in the rumen fluid. Nitrite is absorbed into the blood stream where it facilitates the conversion of hemoglobin to methomoglobin (Kemp et al., 1976; 1977; 1978; Geurink et al., 1979; Malestein et al., 1979; van Broekhoven, 1978). Methemoglobin lacks the ability to transport oxygen throughout the animal's body causing the animal to suffer asphyxation. Nitrite toxicity has also been implicated as one of the causes of abortions in pregnant dairy and beef cows (Simon et al., 1958; Hibbs et al., 1978; Abbit, 1982; Hudson and Rawls, 1992). It is known that the formation of nitrite by the rumen microbes depends closely on the enzymatic activity of nitrate reductase. Two

approaches have been utilized to decrease the levels of toxic nitrite within the animal. One approach attempts to decrease the reduction of nitrate to nitrite by affecting the synthesis or activity of the nitrate reductase. Korzeniowski and researchers examined the effects of tungsten, a molybdenum binding agent, on the nitrate reductase. In both cases tungsten in the form of sodium tungstate was found to have a significant effect on nitrite levels, causing a decrease in the molybdenum containing nitrate reductase activity (Korzeniowski et al., 1980; 1981). The other approach is to increase the breakdown of nitrite. It is this latter approach that has been examined by researchers with two different techniques. The feeding of supplemental concentrates has been confirmed to have a positive effect on the increased rate of ruminal metabolism of both nitrate and nitrite (Burrows et al., 1987). Concentrates such as corn have been given to provide prophylaxis against nitrate intoxication. As mentioned in an earlier section, in the study by Rehberger et al., (1993) denitrifying propionibacteria were given as a direct fed microbial to provide prophylaxis against toxic nitrite levels. The establishment of an increased level of propionibacteria within the rumen was shown to supply a source of additional nitrite reduction.

Nitrate Reductase

Purpose of Nitrate Reductase

Microorganisms utilize nitrate for two distinct reasons (Stouthamer, 1976; Knowles, 1982). In the first instance nitrate can be used as the sole source of nitrogen to be used in all nitrogen-containing compounds of the microorganisms. This process is called nitrate assimilation, which can occur under both aerobic and anaerobic conditions. The second function utilizes nitrate during anaerobic conditions as a terminal electron

acceptor. This process has been termed nitrate respiration or dissimilatory nitrate reduction. In both of these processes, nitrate reductase reduces nitrate to nitrite. In some cases, under anaerobic conditions, the nitrite can also be used as a terminal electron acceptor. During this process, called denitrification, nitrite is converted to gaseous products such as nitrogen or nitrous oxide.

Differentiation of Nitrate Reductases

There are two types of nitrate reducing enzymes that can be separated by their interaction with chlorate and localization within cell free extracts (Stouthamer, 1976). Both types reduce nitrate and this reduction is inhibited by azide. Nitrate reductase type A can use chlorate as a substrate and is localized in the cytoplasmic membrane fraction of cell free extracts. Nitrate reductase type B, unlike type A is inhibited by the presence of chlorate and has been found to be localized within the cytoplasm. The type A nitrate reductase always plays a role in dissimilatory nitrate reduction, while the type B enzyme has different functions and sensitivities in different bacteria. The type B enzyme has been reported to have a dissimilatory nitrate reducing function in some bacteria and a nutritive function in others (Stouthamer, 1976). The type B enzyme has also been found to be repressed by certain inhibitors such as ammonia in some bacteria (Stouthamer, 1976; van't Riet et al., 1968). This body of work will mainly concentrate on the dissimilatory nitrate reductase (Type A) enzyme due to the focus of the presented investigation.

Comparison of Molecular Weight and Molecular Contents of Nitrate Reductase

It is very difficult to compare the molecular weight, subunit complex, and molecular contents (iron, molybdenum, and sulfide) of various nitrate reductases. This is mainly due to the discrepancies in the literature due to the differences in purification procedures. These variations in MW, subunit complex, and molecular contents are typified examining the data from *Klebsiella aerogenes* (van't Riet and Planta 1969; van't Riet et al., 1975) and *Escherichia coli* (MacGregor et al., 1974; Enoch and Lester, 1974; MacGregor, 1975 a;b; Stouthamer, 1976).

The nitrate reductase of *K. aerogenes* can be isolated in different forms, depending on the method used. There are two main forms, nitrate reductase I and II. Nitrate reductase I can be converted to type II by the process of aging or treatment at pH 9.5 in the presence of deoxycholate and 0.5 M sodium chloride. Sedimentation experiments indicate that both nitrate reductase I and II are present as tetramers, which disassociate to monomers in the presence of deoxycholate. This disassociation is reversible upon the removal of deoxycholate. The nitrate reductase I of *K. aerogenes* has three different subunits in molecular ratio of 1:1:2. The molecular weights of the subunits are 1.17×10^5, 5.7×10^4, 5.2×10^4 daltons respectively. The nitrate reductase II has only the 1.17×10^5 and 5.7×10^4 subunits in equimolar ratios. Apparently the pH 9.5, deoxycholate and 0.5 M NaCl treatment removes both copies of the smallest subunit. The labile nature of the nitrate reductase II suggested to the researchers that the two missing subunits have a structural role within the enzyme.

Experiments revealed that the nitrate reductase of *E. coli* can be solubilized in various ways, including ways so the enzyme is complexed with other membrane proteins. Heat treatment solubilization of the enzyme provides a form free of other membrane proteins (MacGregor et al., 1974). The enzyme has two subunits of equimolar ratio with molecular weights of 1.42×10^5 and 5.8×10^4 daltons. The molecular weight of the enzyme is estimated to be 8×10^5 daltons, so it is assumed to be a tetramer. Notice that molecular weight of the smallest subunit of *E. coli* is the same as the smallest subunit of

nitrate reductase II of *K. aerogenes*. However, the molecular weights of the large subunits are different (Stouthamer, 1976).

Another method of solubilization using deoxycholate produced a nitrate reductase with subunits whose molecular weights were estimated to be 1.55×10^5, 6.3×10^4 and 1.9×10^4 daltons (Enoch and Lester, 1974). This study also revealed that the enzyme contained cytochrome *b* and the smallest subunit contained the heme. A solubilization method using heat treatment and alkaline pH was also used to isolate the *E. coli* nitrate reductase (MacGregor, 1975a;b). The purified enzyme was used to make antibody for nitrate reductase precipitation of triton extracted membrane proteins. The triton extracted enzyme had three subunits whose molecular weights were 1.42×10^5, 6×10^4 and 1.95×10^4 daltons, in molecular weight ratios of 1:1:2 respectively (MacGregor, 1975b). The smallest subunit was identified as the apoprotein cytochrome *b*, which is absent from the heat and alkaline pH treatments (MacGregor, 1975b).

Adding to the variations in form, the nitrate reductase has been solubilized in a manner that is complexed with cytochrome *b* and formate dehydrogenase (Iida and Taninuchi, 1959; Itagaki et al., 1962), the latter being the favored electron donor of *E. coli, in vivo*. Thus the method used to isolate the enzyme will influence the size and number of subunits extracted. This in turn will have a direct effect on measurements of nitrate reductase contents.

However, attempts have been made to measure the metal contents of the form of enzyme isolated. A monomer from the nitrate reductase I of *Klebsiella aerogenes* was said to contain eight iron-sulphur groups and four tightly bound non-heme iron atoms. Nitrate reductase II of this bacteria contained no tightly bound iron atoms (van't Riet et al., 1975). Thus, the tightly bound iron is said to be contained in the low molecular weight subunit of the nitrate reductase which is not found in the nitrate reductase type II.

Both *K. aerogenes* and *M. denitrificans* have been found to contain equal amounts of non-heme iron and acid labile sulfide in the monomers of nitrate reductases I and II

(Forget, 1971). These amounts are double that believed to be contained within *E. coli* (Forget, 1974). Influences of these metals (including molybdenum) have been demonstrated in a number of electron paramagnetic resonance spectrometry studies with *M. denitrificans*, (Forget and Der Vartanian, 1972), *E. coli* (Der Vartanian and Forget, 1975) and *K. aerogenes* (van't Riet et al., 1975). Results proved to be similar among the three bacteria when comparing the roles of the three metals (Stouthamer, 1976).

Role of Molybdate

Studies which have examined the effects of molybdenum on nitrate reductase formation, competitive inhibitors of molybdenum utilization, and mutants affected in molybdate metabolism have concluded that a molybdenum cofactor is required for nitrate reductase activity (Stouthamer, 1976).

The presence of molybdate during bacteria growth has shown to increase the specific activity of nitrate reductase (Lester and Demoss, 1971; Enoch and Lester, 1972). Thiocyanate, dithiol, and tungstate demonstrated inhibitory action on the nitrate reductase (Lam and Nicholas, 1969; Radcliffe and Nicholas, 1970; van't Riet et al., 1975). Mutants that are affected in molybdate metabolism have negative effects on nitrate reductase synthesis and activity (Glaser and DeMoss, 1971; van Hartingsveldt and Stouthamer, 1973; Arnst et al., 1970).

Regulation of Dissimilatory Nitrate Reductase Synthesis and Activity

Dissimilatory nitrate reductase synthesis and activity are affected in similar ways by conditions such as the presence of oxygen. Some of the generalities and exceptions are noted in this section, as well as mentioning some substrates than induce the synthesis of the enzyme. It has been stated that when a culture of *K. aerogenes* is taken from anaerobic conditions to aerobic conditions the three following steps occur: i) a stop in nitrate reductase synthesis ii) a stop in nitrate reduction iii) and partial inactivation of the nitrate reductase already present (van't Riet et al., 1968).

The type A nitrate reductase is inducible, but generally requires the absence of oxygen and the presence of nitrate. However, some bacteria such as *Bacillus licheniformis* (Schulp and Stouthamer, 1970) and *Haemophilus influenzae* (Sinclair and White, 1970) only require the absence of oxygen. Bacteria growth with oxygen represses nitrate reductase synthesis and activity. It has been hypothesized that when a culture is switched to anaerobic conditions, the expression of the enzyme is derepressed (de Groot and Stouthamer, 1970a). This follows logically, because the role of nitrate reductase is to enable nitrate to serve as an alternative electron acceptor to oxygen. However in some cases, as with *Bacillus licheniformis*, a change from anaerobic to aerobic conditions only stopped synthesis of nitrate reductase without affecting previously synthesized enzyme (Schulp, 1972). Nitrate reductase activity continued 45-60 minutes after the condition shift.

There are various hypotheses about how aerobic conditions regulate nitrate synthesis and activity. Some findings have suggested that higher energy-yielding potential of oxygen may be what regulates nitrate reductase formation. For example, in *P. mirabilis*, reductases for certain substrates are repressed in the presence of higher energy yielding electron acceptors (de Groot and Stouthamer, 1969; 1970a). When oxygen is present the synthesis of nitrate reductase (nitrate having the lower energy yield) is repressed. Additionally, the presence of oxygen or nitrate represses synthesis of tetrathionate reductase, tetrathionate providing the least potential energy yield of the

three. Following suit is the synthesis of fumarate reductase in *E. coli*. This enzyme is repressed by the presence of oxygen and nitrate (Wimpenny and Cole, 1967; Cole and Wimpenny, 1968). This process of regulation ensures the most amount of energy is released during catabolism (Stouthamer, 1976).

It has also been suggested that the redox potential of the medium, rather than the presence of certain terminal electron acceptors is responsible for the regulation of reductase formation (Wimpenny and Cole, 1967; Wimpenny, 1969). Some researchers also believe that nitrate reductase synthesis is regulated by a nitrate sensitive repressor and a redox sensitive repressor (Showe and Demoss, 1968). It has been proposed by De Groot and Stouthamer (1970 a;b) that the factor regulating the synthesis of reductases is the oxidation-reduction state of the components of the respiratory chain. This hypothesis is in agreement with studies by Simoni and Shalenberger (1972) in *E. coli* and Gilmour et al., (1964) with *P. stutzeri*. Mutants of *E. coli* have demonstrated the ability to synthesize functional nitrate reductase in the presence of oxygen, if electron transport to oxygen is restricted. With *P. stutzeri* it was demonstrated that simultaneous transport to oxygen and nitrate could occur when the oxygen supply is limited. A study using *K. aerogenes* demonstrated the oxidative phosphorylation efficiencies are the same with oxygen or nitrate as the terminal electron acceptor (Hadjipetrou and Stouthamer, 1965; Stouthamer, 1967) This is in agreement with results from using glucose limited chemostat cultures of *K. aerogenes*. The molar growth yield was almost the same in aerobic cultures which contained no nitrate and oxygen limited cultures which metabolized nitrate (Stouthamer, 1976)

Cytochrome *a* research with *B. stearothermophilus* and *B. licheniformis* indicates that anaerobiosis, not the effects of nitrate respiration, is responsible for cytochrome *a* disappearance (Downey and Kiszkiss, 1969; Schulp and Stouthamer, 1970). Nitrate reduction continues until cytochrome *a* production has reached maximum levels. As cytochrome *a* levels increase, an increased electron transport to oxygen was shown. Thus

as seen with nitrate reductase synthesis, nitrate reductase activity continues until a normal level of electron transport to oxygen has been reached, inhibition of nitrate reductase not being due to a direct effect of the presence of oxygen.

A strong hypothesis concerning inactivation of nitrate reductase has been proposed by de Groot and Stouthamer (1970a). They suggest that electron withdrawal from the nitrate reductase to other components of the electron transfer system is responsible for the enzyme inactivation. Thus oxidation is responsible for the inactivation. Inactivation of nitrate reductase in cell free extracts of *K. aerogenes* (van't Riet et al., 1968) and non-inactivation of purified enzyme in the presence of oxygen (van't Riet et al., 1970), support the researcher's hypothesis. These and other observations suggest that nitrate reductase is sensitive to oxygen only in the presence of a functional respiratory chain.

As mentioned previously it has been demonstrated that nitrate in the absence of oxygen can induce nitrate reductase synthesis. However, it was also mentioned that there were two distinct cases where only the absence of oxygen was required for induction (Schulp and Stouthamer, 1970; Sinclair and White, 1970). It should be noted that in both the presence of nitrate did however increase nitrate reductase synthesis. Other than nitrate, electron acceptors nitrite and azide are able to induce synthesis of nitrate reductase under anaerobic conditions (de Groot and Stouthamer 1970a; Chippaux and Pichinoty, 1970).

E. coli Nitrate Reductase Structure

Nitrate reductase of *Escherichia coli* has been extensivly studied providing a model of structure, function and regulation of a membrane bound enzyme. The nitrate reductase of *E. coli* is an enzyme of approximately 230,000 KDa in molecular weight.

This enzyme complex consists of three polypeptide subunits. These are denoted as the alpha, beta, and gamma subunits or subunits A, B, and C respectively (MacGregor, 1975b; Enoch and Lester, 1974;1975; Clegg, 1976). Surface labeling studies have shown that the large subunit A is exposed to the cytoplasm, the smallest subunit C is exposed to the periplasm, and the third subunit B cannot be labeled and is presumed to be buried within the membrane (Garland, 1975; MacGregor and Christoper; 1978). One study examined the fate of pulse-labeled enzyme in both membrane and cytoplasm (MacGregor and MacElhaney, 1981). The results indicated that immediately after insertion of the enzyme into the membrane, a post translational event occurs. The cytoplasmic synthesized form of subunit B is converted to a form found in the completely assembled enzyme.

The alpha subunit (approximately 145 kDa) is believed to contain the nitrate reducing catalytic site of the enzyme (MacGregor, 1975b; Graham and Boxer, 1980a; Chaudry and MacGregor, 1983a; DeMoss, 1977; Stewart, 1988). Proteolysis examination of nitrate reductase provided three main observations that indicated the catalytic site was contained within the A subunit (MacGregor, 1975a). Purified enzyme exhibited nitrate reducing activity independent of subunit B degradation. Active cytoplasmic nitrate reductase contained only the A subunit. The specific activity of cytoplasmic nitrate reductase was significantly higher than the specific acitivity of membrane bound nitrate reductase. Trypsin treatment of nitrate reductase converts the enzyme to a new form containing the alpha-subunit and a 43,000 MW. fragment of the beta subunit (termed B'), which is still enzymatically active when assayed with the artifical electron donor reduced benzyl viologen (DeMoss, 1977). This provides another indication of a catalytic site within the alpha subunit. Graham and Boxer (1980) measured NO_3 reduction of a form of the enzyme which exhibited a modified form of the beta subunit. This modified form of the enzyme was still unaffected in nitrate reducing capacity. One study attempted to determine the location of the catalytic residues by two different methods (Chaudry and MacGregor, 1983). The first method distinguished which subunit is associated with the

molybdenum cofactor, which had been shown to be essential for nitrate reduction. Nitrate reductase activity was restored in non-active A subunits when dialyzed in the presence of molybdenum cofactor, while subunit B exhibited no enzyme activity restoration. In the second experiment dissection and analysis of the individual subunits was utilized to determine the nitrate reducing subunit. Individual subunit A contained the majority of all molybdenum and iron. The dissapperance of the two elements consequently brought about a loss in nitrate reducing activity. These observations coupled with proteolysis and amino acid analyses provided evidence indicating subunit A contains the active site of the nitrate reductase enzyme.

The beta subunit (approximately 60 kDa) function has not been fully ascertained but it is believed to be involved in the attachment of the whole enzyme complex to the membrane (MacGregor, 1975a; 1975b; Chaudry and MacGregor, 1983a; DeMoss et al., 1981; MacGregor and MacElhaney, 1981; Stewart, 1988; Graham and Boxer, 1980b). MacGregor (1975a) theorized that the fact that nitrate reductase activity is higher after heat treatment than before was due to the enzyme being partially buried in the membrane preventing electron donation from methyl viologen from reaching the active site of the enzyme. The study examined pure and partially purified heat extracted enzyme preparations and found that there was a greater than 1:1 ratio of subunit A to B in purified preparations. This indicated that the B subunit was involved in binding to the membrane, leading to the lower amount of B subunit which is often removed with the membrane during purification. Thus, when sufficient amount of enzyme was cleaved due to cell proteolysis the enzyme was released. Additionally, when nitrate reductase activity is compared in particulate and soluble fractions after breakage with a French press, the distribution of activity between the two fractions is equal, further implicating the B subunit function in membrane attachment. These conclusions are supported in examination of mutants with non-functional heme and lacking the cytochrome *b*. Cytoplasm contains undegraded B subunits unlike mutants that lack the cytochrome *b* and exhibit degradation

of the B subunit. It was also found that the *hem*A mutants had 25% more of the A subunit than B. MacGregor interpreted this in the following way: if all of the nitrate reductase synthesized was inserted into the membrane than this membrane would consist of approximately 32% nitrate reductase which most likely would not occur. Thus the uninserted B subunit goes undegraded and the excess A subunit present is released from the membrane, thereby providing the higher amount of subunit A observed.

Structural changes which occur upon purification of nitrate reductase have indicated to researchers that the carboxyl terminal segment of the B subunit is involved in the binding of the enzyme to cytochrome *b* and its association with the membrane (DeMoss et al., 1981). During early purification stages cytochrome *b* disassociated from the enzyme and a 2000 dalton segment was removed from the carboxyl terminal end. This isolated 2000 dalton fragment was shown to be cleaved by trypsin but was unaffected in earlier treatments when the enzyme was associated with the cell. This is explained by two possibilities: release of the enzyme from previously protected membrane bound domains renders the enzyme susceptible to trypsin cleavage, indicating this region may have been responsible for the interaction of the enzyme with the membrane. Alternatively, this segment may be involved with the binding to cytochrome *b*. This was demonstrated by the fact that the subunit B did not become the modifed form of subnit B until after cytochrome *b* had dissociated from the enzyme. This hypothesis is in agreement with an earlier proposal that in *E. coli* binding of nitrate reductase to the membrane involves an interaction with membrane bound cytochrome *b* (MacGregor, 1975b). Evidence was later provided that a more rapidly migrating form of the 60,000 dalton subunit B is synthesized in the cytoplasm and then binds to the membrane (MacGregor and McElhaney, 1981). The lack of labeling of subunit B by either transglutuminase dansyl cadaverine or $[^{125}]$I lactoperoxidase suggested that the subunit may be protected within the lipid bilayer. Thus it is likely the subunit is involved in membrane binding and possibily may be the subunit which interacts with cytochrome *b*. It was later theorized to be an electron transfer unit

that contains and/or contributes 3 or 4 iron-sulfur centers, (Blasco et al., 1989) as deduced from amino acid sequencing. This is contrary to popular theory of the alpha subunit being that which binds the iron for the alpha-beta complex based on EPR studies (Chaudry and MacGregor, 1983a).

The gamma subunit (approximately 20-25.5 kDa) is the apoprotein of the b-type cytochrome of *E. coli*. This subunit works specifically to transfer electrons to the alpha-beta complex (Ruiz Herrera and DeMoss, 1969b; Chaudry and MacGregor, 1983b). Early examinations of the cytochrome *b* indicated that the apoprotein was directly involved in nitrate reduction by accepting electrons from formate dehydrogenase and in turn transferring the electrons to nitrate reductase (Iida and Taniquchi, 1959; Itagaki and Fujita, 1969; Ruiz Herrera and DeMoss, 1969a; Wimpenny and Cole, 1967; Ruiz-Herrera and DeMoss, 1969b). Enoch and Lester suggested that the heme may be associated with subunit C, finding that no heme had been detected in earlier isolations of nitrate reductase, which were cytochrome *b* free (1974). MacGregor demonstrated that the small subunit C of nitrate reductase was actually the cytochrome *b* apoprotein (1975b). Spectral study of cytochrome *b* provided the first line of evidence. The following evidence was given: (i) anti-nitrate reductase serum precipitates cytochrome *b* while preimmune serum does not; (ii) from these extracts cytochrome *b* precipitation occurs with nitrate reductase precipitation; (iii) decreasing amounts of enzyme precipitation occurs simutaneously as cytochrome *b* precipitation decreases. The second line of evidence was provided by *hem*A mutant experimentation. Mutants that lack the ability to synthesize heme still produce nitrate reductase however the antibody precipitated enzyme contains no subunit C. This study also demonstrated the involvement of cytochrome *b* in the regulation of nitrate reductase, this regulation occuring in two ways, regulating the amount of enzyme produced and the attachment of the enzyme to the membrane. Mutants lacking the cytochrome *b* subunit exhibited a 1.6 fold increased production of nitrate reductase than the wild type. Additionally, SDS gels indicate that these mutants show degradation of the

B subunit, which is not demonstrated in the wild type containing the cytochrome *b*. Further evidence demonstrated a loss of the B subunit and a reduction in the total amount of membrane bound nitrate reductase. Cytochrome b was found to be involved in binding the precursor forms of subunits A and B to the membrane *in vivo* (Chaudry and MacGregor, 1983b). *In vitro* examinations in this study further indicated that cytochrome *b* prevents the modification of the B subunit. The cytochrome *b* subunit was also suggested to have a stronger association with nitrate reductase than previously believed. It was once thought that cytochrome removal and release of the enzyme from the membrane was due to proteolytic process (MacGregor, 1975a). Researchers later found this hypothesis to be wrong finding that isolated cytochrome *b* was very resistant to proteolytic treatment showing sensitivity only to trypsin at one site on the molecule. Cytochrome *b* did not dissassociate easily from the purified enzyme during storage or during treatment with non-denaturing detergents or chaotropic agents. Only low concentrations of SDS could cause dissassociation without complete denaturation (Chaudry and MacGregor, 1983b).

Escherichia coli Nitrate Reductase

Structural Genes

The alpha, beta, and gamma subunits are encoded by the structural genes *nar*G, *nar*H, and *nar*I. From the nucleotide sequence of *nar*G, the gene is predicted to encode a polypeptide of 1239 amino acids with the molecular weight of 138,682 daltons (Blasco, 1989; MacPherson et al., 1984). This is in agreement with values given for the molecular weight of the A subunit based on SDS-PAGE estimations (Stewart, 1988). Hydropathy analysis revealed no distinct domains of marked hydropathy. The *nar*H gene product is a polypeptide of 512 amino acids with a molecular weight of 57,751 daltons as deduced from nucleotide sequence (Blasco, 1989) which is also in good agreement with previously

cited values (Stewart, 1988). It was later found that the *narI* structural gene contained 2 open reading frames. The newly discovered gene was designated as *narJ*, which produces a polypeptide that is 25.5 KDa in molecular weight. These 4 structural genes constitute a 7 Kb transcriptional unit with the order *nar*G-H-J-I (Bonnefoy-Orth et al., 1981; Edwards et al., 1983; Sodergren and DeMoss, 1988; Stewart and MacGregor, 1982). This operon is located at 27 minutes on the *E. coli* chromosomal map.

Nucleotide and amino acid sequencing of the the *nar* operon revealed that there are five regions of high homology sequences between the largest subunits of other *E. coli* oxioreductase molybdoenzymes examined and nitrate reductase (Blasco, 1989; Weiner et al., 1988). These regions are thought to be involved in the binding of the molybdenum cofactor to the enzyme (Bilous et al., 1988). These results provide genetic evidence that the *E. coli* nitrate reductase is a molydoenzyme. Biochemical studies demonstrated that the *E. coli* nitrate reductase requires molybdenum cofactor, because it was found that in the absence of molybdenum there is no nitrate reductase activity. Thus the nitrate reductase was classified as a molybdoenzyme (Stewart, 1988).

Escherichia coli Nitrate Reductase Regulation

The general observations concerning nitrate reductase regulation in *E. coli* apply to most bacteria. There are many factors and conditions that regulate nitrate reductase activity and synthesis. Aerobic cultures have extremely low nitrate reductase activity, while anaerobic cultures have significant activity (Showe and DeMoss, 1968). The addition of nitrate increases enzyme activity 20 fold in an anaerobic culture. Nitrate has no effect in an aerobically grown culture. These observations suggested that there is a two step induction that results from two separate regulatory signals, one from a repressor sensitive to nitrate and the other signal from a redox sensitive repressor (Showe and

DeMoss, 1968; Stewart 1982). This hypothesis was later shown to be accurate using *chl*C-lac operon fusions to study regulatory mutations which affect nitrate reductase expression in *Escherichia coli*. Anaerobic formation of nitrate reductase is under the control of the *fnr* gene, while the nitrate induction of nitrate reductase is under the control of the *nar*L gene (Stewart, 1982). *Fnr* mutations resulted in a complete lack of induction, whereas the narL- mutants lacked only the second, nitrate-specific step of induction. These two observations present genetic evidence to support the two step model for induction of nitrate reductase.

The gene product of the *fnr* gene is the positive regulator for the synthesis of several anaerobic enzymes (Chippaux et al., 1982; Lambden and Guest, 1976). The Fnr protein is rendered inactive during aerobic conditions, but is activated upon anaerobiosis (Shaw and Guest, 1983). The nucleotide sequence of this enzyme that controls anaerobic expression of the nitrate reductase operon has been determined. It is located approximately 110 bp upstream from the *nar*G transcriptional initiation site (50 bp upstream of the translational start site) (Li and DeMoss, 1988). It has been shown that the deduced amino acid sequence of Fnr has a high degree of homology to Cap (Crp), the regulatory protein mediating catabolite repression (Spiro and Guest, 1990). This study also revealed regions in fnr involved in the binding of Fe^{2+} or Fe^{3+}. It was suggested that the oxidation of Fe^{2+}-protein complex to the Fe^{3+}-protein complex by O_2 leaves Fnr nonfunctional. This follows logically considering that all operons depend on the Fnr activator, such as the nitrate and fumarate reductase operons, are not expressed during aerobic conditions.

As indicated, nitrate induction requires the *nar*L gene product. This protein not only induces nitrate reductase synthesis but also represses some of the other terminal electron acceptor enzyme's synthesis (Iuchi and Lin, 1987). It was later found that two genes, *nar*L and a *nar*X gene mediate nitrate reductase synthesis in the presence of nitrate and nitrate repression of fumarate reductase synthesis (Stewart et al., 1989). This was

even further clarified when research indicated that in the presence of nitrate and molybdenum, the *nar*X gene product modifies or "activates" the *nar*L gene product. Thus it is proposed that the function of the *nar*X protein is as a sensor for the availability of nitrate and possibly molybendum (Kalman and Gunsalus, 1990). The *nar*L and *nar*X genes encode for proteins of 23 KDa and 66 KDa respectively. Both genes have been cloned, sequenced (Kalman and Gunsalus, 1989; Nohno et al., 1989; Stewart et al., 1989) and have been located adjacent to each other at 27 minutes on the chromosomal map of *E. coli*, upstream from the structural *nar*G gene (Kalman and Gunsalus, 1988; Kalman and Gunsalus, 1989; Stewart, 1982; Stewart and Parales, 1988). Two-thirds of the N-terminal end of the *nar*L protein has extensive homology with many diverse prokaryotic regulatory proteins including OmpR, PhoB, SfrA, UhpA, CheY, CheB, NtrC, DctD, FixJ, VirG, SpoOF, and SpoOA (Nohno et al., 1989; Noji et al., 1989). A segment of the C-terminal end of the protein appears to have the most similarity to regions that form the helix-turn-helix motif of DNA-binding proteins. The narX protein has regions on the C-terminal end with homology to the counterparts of two-component regulatory system proteins including EnvZ, PhoR, PhoM, CpxA, NtrB, DctB, FixL, and VirA. Hydrophobic regions within the *nar*X product N-terminal half suggest a function as a transmembrane receptor sensing nitrate.

Another gene, *nar*K, plays a role in nitrate reductase regulation. The *nar*K gene product, a transmembrane protein participates in nitrate transport. Noji and researchers (1989) suggest that the *nar*K gene encodes a nitrate/nitrite antiporter which enhances the ability of the cells to transport nitrate and nitrate across the membrane of the cell. This would facilitate anaerobic nitrate respiration serving as a high-affinity nitrate uptake system as nitrate is being converted to nitrite. The nitrate concentration gradient is then used to drive nitrite from the cell as rapidly as it is formed. This also helps to maintain the electrogenic balance of the cell due to nitrate uptake being associated with nitrite excretion. These hypotheses were verified in further mutational examinations of the *nar*K

gene by DeMoss and Hsu (1991). The *nar*K gene including regulatory promoter regions has been sequenced. Five Cys residues in the *nar*K gene were found and are suggested to play a vital part in sensing redox potential to regulate the nitrate/nitrite facilitator. Regulatory promoter regions of the gene were found to contain "nitrate box" and an "anaero-box" sequences homologous to *nar*GHJI promoter regions (Noji et al., 1989).

The following model for nitrate reductase is proposed. The NarX protein (nitrate sensor) in the presence of nitrate is activated and converts the NarL protein to an active form that binds to the " nitrate box" of the *nar*K gene. Meanwhile during anaerobic conditions, the Fnr protein binds the "anaero-box". These two mechanisms promote the synthesis of the *nar*K gene product, a transmembrane protein that participates in nitrate transport (Noji et al., 1989).

A gene designated *nar*Q has been found to be a nitrate sensor that co-functions in signal transduction reactions with nitrate sensor *nar*X (Rabin and Stewart, 1992b; Chiang, 1992). Results indicated that the *nar*X/*nar*Q-*nar*L regulatory system represents neither "cross-talk" nor "cross regulation", but rather a situation existing where either sensor is sufficient for normal regulation by a single physiological signal (nitrate). The *nar*Q gene mapped to 53 minutes on the *E. coli* genetic map, a location distinct from all known regulatory or target genes. The predicted *nar*Q sequences reveals substantial similarity to *nar*X and other membrane associated histidine protein kinase sensors. These observations suggest *nar*X and *nar*Q are functionally redundant for nitrate signalling to *nar*L.

A second response regulator designated *nar*P has been found to be involved in nitrate regulation with *nar*L (Rabin and Stewart, 1993). Mutational experiments provided genetic evidence that either *nar*X or *nar*Q proteins can interact with *nar*L and *nar*P proteins to regulate target operon expression in response to nitrate. Additionally, it was demonstrated that nitrite, also acts to control target operon expression via the *nar*X, *nar*Q, *nar*L and *nar*P proteins. Nitrite was found to be the primary signal inducing the *nar*P operon. In contrast, nitrite is a relatively weak inducer of the *nar*L dependent

operon. It is suggested that the *nar*Q protein communicates the presence of nitrite to both the *nar*P and *nar*L proteins and that the *nar*X proteins inhibits this communication with the *nar*L protien.

Another protein, Integrating Host Factor (IHF), has been found to be essential for induction of the nitrate reductase operon. Observations made during earlier studies with *E. coli* led to the discovery of IHF. If the *nar*L binding sequence is moved by more than 10 base pairs or is inverted, than nitrate induction of the *nar* operon will not occur (Li and Demoss, 1988; Rabin et al., 1992; Dong et al., 1992). Additionally altering the composition or location of the DNA sequences between the *nar*L- and *fnr*-binding sequences will also abolish nitrate induction (Dong, 1992). These observations suggested that the formation of a specific nucleoprotein structure was required for the interaction of *nar*L with the *nar* operon, rather than a simple loop mechanism. Rabin and researchers have demonstrated that the IHF function is required for nitrate induction of *nar* operon expression *in vivo* (Rabin et al.,1992). DNase I footprint and gel-mobility shift experiments revealed a single site for IHF binding in the *nar* operon control regions. This site was located midway between the upstream *nar*L binding site and the transcription initiation region. It is suggested the IHF mediates a specific loop structure that brings the *nar*L-binding sequence into correct proximity to the transcriptional initiation site.

Escherichia coli Contains a Second Nitrate Reductase

A second nitrate reductase, designated NRZ, which when overproduced, substituted for the more characterized *nar*GHJI gene (NRA) in all respects examined (Bonnefoy et al., 1987). It was later found that the NRZ enzyme was membrane bound and produced 3 subunits that correspond to the NRA enzyme (Iobbi et al., 1987). Purified nitrate reductases A and Z were similar exhibiting the same molecular mass, subunit

composition and utilized the same electron donors and acceptors. The two enzymes did differ in electrophoretic migrations and susceptibility to trypsin (Iobbi-Nivol, 1990). The NRZ DNA sequence has been deduced, and is organized in the transcription unit designated *nar*ZYWV. These genes are 73% homologous to the *nar*GHJI genes. The NRZ structural genes located on a 14 Kb fragment at 32.5 min. on the chromosome of *E. coli* (Blasco et al., 1990). The expression of the chromosomal copy of the NRZ genes appear to be negatively controlled by the Fnr protein during anaerobic conditions. However, when the NRZ genes are cloned into a multicopy plasmid, expression is totally independent of both Fnr and nitrate (Bonnefoy et al., 1987), contrary to NRA gene research results (Stewart, 1988). These results were inconclusive because the possibility existed that the differences were due to the absence of regulatory elements on the cloned gene or titration of these elements during high gene concentrations. Anti-serum studies led to the conclusion that the two enzymes expression are regulated oppositely (Iobbi-Nivol, 1990). The *nar*GHJI operon is aerobically repressed, strongly induced by nitrate and positively regulated by the Fnr gene product (Showe and DeMoss, 1968; Chippaux et al., 1982). The NarZ enzyme expression is repressed under anaerobic conditions, shows weak to no induction by nitrate and is negatively regulated by the *fnr* gene product (Iobbi-Nivol, 1990). Upstream from the *narZ* operon there exists an open reading frame that is 73% homologous to the C-terminal end of the *nar*K gene (Blasco et al., 1990). However, this region is approximately 80 nucleotides long and does not have the *cis*-acting elements, *nar*L and *fnr* boxes, nor the *ter*C4 terminator sequence present in the 500 nucleotide *nar*K-*nar*G intergenic region. This possibily explains the marked differences in enzyme regulation. The researchers suggest that these results indicate that a fragment larger than the *nar*GHJI operon has been duplicated. It was suggested that the NRZ exists to catalyze the immediate flow of electrons to nitrate during aerobic/anerobic shifts when grown in the presence of nitrate. Nitrate reductase A is then subsequently produced (Iobbi-Nivol, 1990).

Nitrate Reductase of Propionibacteria

Although propionibacteria have not been studied as extensively as *E. coli*, a number of studies have been performed providing some insight to the nature of propionibacteria dissimilatory nitrate reduction. Sone suggested that cytochrome *b* plays a major role in *P. arabinosum* anaerobic respiration (Sone, 1972). The study demonstrated in part the nature of the redox enzymes involved in the bacteria's electron transfer system. Further examination of the cytochromes of propionibacteria continued as researchers identified the effects of oxygen upon cytochrome synthesis (De Vries et al., 1972). Strains *P. freudenreichii, P. shermanii, P. rubrum* , and *P. pentosaceum* were studied to determine the effects on propionibacteria grown in the presence of oxygen. In all strains of propionibacteria studied, cytochromes *b, a* or a_1, and a_2 were synthesized. Strains *P. freudenreichii* and *P. shermanii* exhibited growth and cytochrome synthesis inhibition in the presence of oxygen. Accordingly cytochrome synthesis paralleled cell growth. Strains *P. pentosaceum* and *P. rubrum* grew slightly faster in aerobic conditions than anaerobic, and cytochrome synthesis was less inhibited than with the other two species. Using HOQNO (an inhibitor of cytochrome *b*) it was also demonstrated that cytochrome *b* functions directly in anaerobic electron transport from lactate to fumarate. Oxidative phosphorylation was the only possible way to gain energy during aerobic conditions. The influence of nitrate upon cythochrome *b* in *P. pentosaceum* has been examined by van Gent-Ruijters and researchers (1975). One of three strains of *P. pentosaceum* appeared to reduce nitrate under anaerobic conditions. Nitrate was reduced to nitrite and when nitrate was exhausted, nitrite was reduced further to presumably either N_2 or N_2O. Cytochrome b functioned in the transfer of electrons from lactate glycerol I phosphate and NADH to nitrate. Cytochrome *b* synthesis did not appear to be repressed by oxygen when cells were grown in the presence of nitrate. Nitrite had a toxic effect to cell cultures, inhibiting growth at high levels. Molar growth yields increased in the presence of nitrate indicating

increased ATP production. This was suggested to be explained by citric acid cycle activity and oxidative phosphorylation coupled to nitrate reduction.

The *P. acidipropionici* (formerly named *P. pentosaceum*) nitrate reductase has been partially purified, and estimated to have a molecular weight of 230,000 daltons (Kaneko and Ishimoto, 1978). This molecular weight is the approxiamte size of the nitrate reductase found in *Escherichia coli* (MacGregor, 1975b; Enoch and Lester, 1974;1975; Clegg, 1976), *Bacillus halodenitrificans* (Ketchum et al., 1991) and *Pseudomonas denitrificans* (Ishozika et al., 1984). Glycerol phosphate, NADH, and lactate were shown to serve as electron donors of nitrate reductase activity. Nitrate reductase had a pH optimum of 6.5-7.5 and used benzyl and methylviologen as artificial electron donors. Tungstate lowered nitrate reductase activity, which was restored upon the addition of molybdate. These results suggested that the *P. acidipropionici* nitrate reductase was a molybdo-protein similar to the nitrate reductase of other bacteria. Kasper examined the presence of a nitrite reducing enzyme in various strains of propionibacteria (Kaspar, 1982). All strains studied reduced nitrate to nitrite which was further reduced to nitrous oxide. Further reduction of nitrous oxide could not be found. Oxygen inhibited N_2O production in strains of *P. acidipropionici* and *P. theonii*. While growth in the presence of nitrate stimulated the production of N_2O in all strains. Kaspar suggested that the enzymes involved in nitrate and nitrite reduction were either constitutive or derepressed. Some results also indicated the possibility of an incomplete form of dissimilatory nitrate reduction to ammonia. As with other studies high levels of nitrite was toxic to cell cultures, suggesting that nitrite reduction occurs as a detoxification mechanism rather than a part of an energy transformation system. Nitrate reduction rates were generally lower than nitrite reduction rates. Dissimilatory nitrate reduction has been also examined in the cutaneous strain *P. acnes* (Allison and MacFarlane, 1989). When grown in the presence of nitrate or nitrite a particulate nitrate reductase and a soluble nitrite reductase was produced. A nitrous oxide reducing enzyme could not be detected.

Culture pH strongly influenced products of dissimilatory nitrate reduction. Nitrate was predominately reduced to nitrite at alkaline pH, while nitrous oxide was the main product produced at pH 6.0. Reduced viologen dyes were the preferred electron donors of these enzymes. Results of nitrite inhibition upon cell culture growth further inferred the nitrite reductase function as a detoxification mechanism as suggested in other propionibacteria studies.

References

Abbit, B. 1982. A case of nitrate induced abortion in cattle. Southwestern Vet. **35**:12.

Allison, C., and G. MacFarlane. 1989. Dissimilatory nitrate reduction by *Propionibacterium acnes*. Appl. Environ. Microbiol. **55**:2899-2903.

Anderson, T. 1985. Mycostatic whey and process of manufacture. U.S patent 4,497,833.

Antipov, V., and V. Subbotin. 1980. Use of probiotics-efficacy and prospects. Ref. Dairy Sci. Abstr. 1982, 44(11):8567.

Arnst, H. Jr., D. MacDonald, and D. Cove. 1970. Molybdate metabolism in *Aspergillus nidulans*. I. Mutations affecting nitrate reductase and/or xanthine dehydrogenase. Mol. Gen. Genet. **108**:129-145.

Biede, S., and E. Hammond. 1979. Swiss cheese flavor. II. Organoleptic analysis. J. Dairy Sci. **62**:238-248.

Bilous, P., S. Cole, W. Anderson, and J. Weiner. 1988. Nucleotide sequence of the *dms*ABC operon encoding the anaerobic dimethylsulfoxide reduction of *Escherichia coli*. Mol. Microbiol. **2**:785-795.

Blasco, F., C. Iobbi, G. Giordano, M. Chippaux, and V. Bonnefoy. 1989. Nitrate reductase of *Escherichia coli*: Completion of the nucleotide sequence of the *nar* operon and reassessment of the role of the alpha and beta subunits in iron binding and electron transfer. Mol. Gen. Genet. **218**:249-256.

Blasco, F., C. Iobbi, J. Ratouchniak, V. Bonnefoy, and G. Chippaux. 1990. Nitrate reductases of *Escherichia coli*: Sequence of the second nitrate reductase and comparison with that encoded by the *nar*GHJI operon. Mol. Gen. Genet. **222**:104-111.

Bonnefoy, V., J. Burini, G. Giordano, M. Pascal, and M. Chippaux. 1987. Presence in the "silent" terminus region of the *Escherichia coli* K12 chromosome of cryptic gene(s) encoding a new nitrate reductase. Mol. Microbiol. **1**:143-150.

Bonnefoy-Orth, V., M. Lepelletier, M. Pascal, and Chippaux, M. 1981. Nitrate reductase and cytochrome $b_{\text{nitrate reductase}}$ structural genes as parts of the nitrate reductase operon. Mol. Gen. Genet. **181**:535-540.

Broekhoven, L., and R. Stephany. 1978. Environmental aspects of N-nitroso compounds. IARC Scientific Publications: 19.

Brumm, P., and R. Datta. 1989. Production of organic acids by an improved fermentation process. U.S patent 4,814,273.

Burrows, G., G. Horn, R. McNew, L. Croy, R. Keeton, and J. Kyle. 1987. The prophylactic effect of corn supplementation on experimental nitrate intoxication in cattle. J. Anim. Sci. **64**:1682-1689.

Cerna, J. 1984. Elvit, fermented milk beverage. Prum.-Potravin. **35**:192-196.

Chaudhry, G., and C. MacGregor. 1983a. *Escherichia coli* nitrate reductase subunit A: its role as the catalytic site and evidence for its modification. J. Bacteriol. **154**:387-394.

Chaudhry, G., and C. MacGregor. 1983b. Cytochrome *b* from *Escherichia coli* nitrate reductase. Its properties and association with the enzyme complex. J. Biol. Chem. **258**:5819-5827.

Chiang, R., C. Cavicchioli, and R. P. Gunsalus. 1992. Identification and characterization of *narQ*, a second nitrate sensor for nitrate-dependent gene regulation in *Escherichia coli*. Mol. Microbiol. **6**:1913-1923.

Chippaux, M., V. Bonnefoy-Orth, J. Ratouchniak, and M. Pascal. 1982. Operon fusion in the nitrate reductase operon and study of the control gene *nir*R in *Escherichia coli*. Mol. Gen. Genet. **182**:477-479.

Chippaux, M., and F. Pichinoty. 1970. Les nitrate-reductases bacteriennes. V. Induction de la biosynthese de l,enzyme A par l'azoture. Archiv fur Mikrobiologie. **71**:361-366.

Clegg, R. 1976. Purification and some properties of nitrate reductase (E.C. 1-7-99-4) from *Escherichia coli* K12. Biochem. J. **153**:533-541.

Crueger, W., and A. Crueger. 1982. Biotechnology: a textbook of industrial microbiology. Science Tech. Inc. Madison, WI.

Cummins, C., and J. Johnson. 1986. Section 15. Propionibacterium, pp. 1346-1353. *In* Peter H. A. Sneath, (ed.); Bergey's Manual of Systematic Bacteriology, Vol. 2. The Williams and Wilkins Co., Baltimore, MD.

Dawson, T., L. Miranda, M. Yokoyama and S. Rust. 1991. Inoculation of reconstituted high moisture corn with propionic acid producing bacteria with or without the additon of lactic acid bacteria. 21st Biennial Conference on Rumen Function. Chicago, IL. November, 1991. (Abst. 62)

DeMoss, J. A. 1977. Limited proteolysis of nitrate reductase purified from membranes from *Escherichia coli*. J. Biol. Chem. **252**:1696-1701.

DeMoss, J., T. Fan, and R. Scott. 1981. Characterization of subunit structural alterations which occur during purification of nitrate reductase from *Escherichia coli*. Arch. Biochem. Biophys. **206**:54-64.

DeMoss, J., and P. Hsu. 1991. NarK enhances nitrate uptake and nitrite excretion in *Escherichia coli*. J. Bacteriol. **173**:3303-3310.

Der Vartanian, D., and P. Forget. 1975. The bacterial nitrate reductase EPR studies on the enzyme A of *Escherichia coli* K12. Biochim. Biophys. Acta. **379**:74-80.

DeVries W., M. Aleem, A. Hemrika-Wagner, and A. Stouthamer. (1977). The functioning of cytochrome b in the electron transport to fumarate in *Propionibacterium freudenreichii* and *P. pentosaceum*. Arch. Microbiol. **112**:271-276.

DeVries, W., W. van Wijck-Kapteign and A. H. Stouthamer. 1972. Influence of oxygen on growth, cytochrome synthesis and fermentation pattern in propionic acid bacteria. J. Gen. Microbiol. **71**:515-524.

Dong, X., J. Li, and J. DeMoss. (1992). Upstream elements required for NarL-mediated activation of transcription from the narGHJI promoter of *Escherichia coli*. J. Biol. Chem. **263**:14122-14128.

Downey, R., and Kiszkiss, D. 1969. Oxygen and nitrate induced modification of the electron transfer system of *Bacillus stearothermophilus*. Microbios. **2**:145-153..

Duwat, P., S. Ehrlich, and A. Gruss. 1992. Use of degenerate primers for polymerase chain reaction cloning and sequencing of the *Lactococcus lactis* subsp. *lactis recA* Gene. Appl. Environ. Microbiol. **58**:2674-2678.

Edwards, E., S. Rondeau, J. DeMoss. 1983. *chlC*(*nar*) operon of *Escherichia coli* includes genes for alpha and beta subunits of the nitrate reductase. J. Bacteriol. **153**:1513-1520.

Enoch, H., and R. Lester. 1972. Effects of molybdate, tungstate, and selenium compounds on formate dehydrogenase and other enzyme systems in *Escherichia coli*. J. Bacteriol. **110**:1032-1040.

Enoch, H., and R. Lester. 1974. The role of a novel cytochrome $_b$ containing nitrate reductase and quinone in the *in vivo* reconstruction of formate-nitrate reductase activity of *Escherichia coli*. Biochem. Biophys. Res. Commun. **61**:1234-1241.

Enoch, H., and R. Lester. 1975. The purification and properties of formate dehydrogenase and nitrate reductase from *Escherichia coli*. J. Biol. Chem. **250**:6653-6705.

Flores-Galarza, R., B. Glatz, J. Bern, and L. van Fossen. 1985. Preservation of high-moisture corn by microbial fermentation. J. Food Prot. **48**:407-411.

Forget, P. 1971. Les nitrate-reductases bacteriennes. Solublisation, purification et proprietes de l'enzyme A de *Micrococcus denitrificans*. Eur. J. Biochem. **18**:442-450.

Forget, P. and D. Der vartanian. 1972. The bacterial nitrate reductases: EPR studies on nitrate reductase A from *Micrococcus denitrificans*. Biochim. Biophys. Acta. **256**:600-606.

Forget, P. 1974. The bacterial nitrate reductases. Solubilization, purification and properties of the enzyme A of *Escherichia coli* K12. Eur. J. Biochem. **42**:325-332.

Garland, P., R. Clegg, D. Boxer, J. Downie, and B. Haddock. 1975. In E. Quagliariello et al. (ed.) Electron transport and oxidative phosphorylation. p. 351-358. North Holland, Amsterdam.

Gent-Ruijters, M. van, van W. de Vries and A. Stouthamer. 1975. Influence of nitrate on fermentation pattern, molar growth yields and synthesis of cytochrome *b* in *Propionibacterium pentosaceum*. J. Gen. Microbiol. **88**:36-48.

Geurink, J., A. Malestein, A. Kemp, and A. van't Klooster. 1979. Nitrate poisoning in cattle. 3. The relationship between nitrate intake with hay or fresh roughage and the speed of intake on the formation of methemoglobin. Neth. J. argic. Sci. **27**:268-276.

Gilmour, C., R. Bhatt, and J. Mayeux. 1964. Comparative role of nitrate and molecular oxygen in the dissimilation of glucose. Nature, London. **203**:55-58.

Glaser, J., and J. DeMoss. 1971. Phenotypic restoration by molybdate of nitrate reductase activity in chlD mutants of *Escherichia coli*. J. Bacteriol. **108**:854-860.

Graham, A., and D. Boxer. 1980a. Implication of alpha-subunit of *Escherichia coli* nitrate reductase in catatlytic activity. Biochem. Soc. Trans. **8**:329-330.

Graham, A., and D. Boxer. 1980b. The membrane location of the beta subunit of nitrate reductase from *Escherichia coli*. Biochem. Soc. Trans. **8**:331.

Grinstead, D., and S. Barefoot. 1992. Jenseniin G, a heat-stable bacterocin produced by *Propionibacterium jensenii* P126. Appl. Environ. Microbiol. **58**:215-220.

Groot, G. de and A. Stouthamer. 1969. Regulation of reductase formation in *Proteus mirabilis*. I. Formation of reductases and enzymes of the formic hydrogenlyase complex in the wild type and in chlorate-resistant mutants. Archiv fur Mikrobiologie. **66**:220-233.

Groot, G. de, and A. Stouthamer. 1970a. Regulation of reductase formation in *Proteus mirabilis*. II. Influence of growth with azide and of haem deficiency on nitrate reductase formation. Biochim. Biophys. Acta. **208**:414-427.

Groot, G. de, and A. Stouthamer. 1970b. Regulation of reductase formation in *Proteus mirabilis*. III. Influence of oxygen, nitrate and azide on thiosulfate reductase and tetrathionate reductase formation. Archiv fur Mikrobiologie. **74**:326-329.

Hadjipetrou, L., and A. Stouthamer. 1965.Energy production during nitrate respiration by *Aerobacter aerogenes*. J. Gen. Microbiol. **38**:29-34.

Hartingsveldt, J. van, and A. Stouthamer. 1973. Mapping and characterization of mutants of *Pseudomonas aeruginosa* affected in nitrate respiration in aerobic or anaerobic growth. J. Gen. Microbiol. **74**:97-106.

Hettinga, D., and G. Reinbold. 1975. Split defect of Swiss cheese. II. Effect of low temperatures on the metabolic activity of *Propionibacterium*. J. Milk Food Technol. **38**:31-35.

Hettinga, D., G. Reinbold, and E. Vedamuthu. 1974. Split defect of Swiss cheese. I. Effect of strain of *Propionibacterium* and wrapping material. J. Milk Food Technol. **37**:322-328.

Hibbs, C. M., E. Stencel, and R. Hill. 1978. Nitrate toxicosis in cattle. Vet. and Human Toxicology. **20**:1-2.

Hofherr, L., and B. Glatz. 1983. Mutagenesis of strains of propionibacteria to produce cold sensitive mutants. J. Dairy Sci. **66**:2482-2487.

Hudson, D., and J. Rawls. 1992. Nitrate toxicity in commercial beef herd. Nebraska Cattleman. **48**:58.

Iida, K., and S. Taniguchi. 1959. Studies on nitrate reductase system of *Escherichia coli*. Particulate electron transport system to nitrate and its solubilization. J. Biochem. **46**:1041-1055.

Iobbi, C., C. Santini, V. Bonnefoy, and G. Giordano. 1987. Biochemical and immunological evidence for a second nitrate reductase in *Escherichia coli* K12. Eur. J. Biochem. **168**:451-459.

Iobbi-Nivol, C., C. Santini, F. Blasco, and G. Giordano. 1990. Purification and characterization of the second nitrate reductase of *Escherichia coli* K-12. J. Biochem. **188**:679-687.

Ishizuka, M., T. Toraya, and S. Fukui. 1984. Purification, properties and limited proteolysis of nitrate reductase from *Pseudomonas denitrificans*. Biochem. Biophys. Acta. **786**:133-143.

Itagaki, E., T. Fujita, and R. Sato. 1962. Solubilization and properties of formate dehydrogenase and cytochrome b_1 from *Escherichia coli*. J. Biochem. **52**:131-141.

Iuchi, S., and E. Lin. 1987. The *nar*L gene product activates the nitrate reductase operon and represses the fumarate reductase and trimethylamineN-oxide reductase operons in *Escherichia coli*. Proc. Natl. Acad. Sci. USA. **84**:3901-3905.

Kalman, L., and R. Gunsalus. 1988. The *frd*R gene of *Escherichia coli* globally regulates several operons involved in anaerobic growth in response to nitrate. J. Bacteriol. **170**:623-629.

Kalman, L., and R. Gunsalus. 1989. Identification of a second gene involved in global regulation of fumarate reductase and other nitrate-controlled genes for anaerobic respiration in *Escherichia coli*. J. Bacteriol. **171**:3810-3816.

Kalman, L., and R. Gunsalus. 1990. Nitrate and molybdenum-independent signal transduction mutations in *nar*X that alter regulation of anaerobic respiratory genes in *Escherichia coli*. J. Bacteriol. **172**:7049-7056.

Kaneko, M., and M. Ishimoto. (1978) A study on nitrate reductase from *Propionibacterium acidi-propionici*. J. Biochem. **83**:191-200.

Kaspar, H. 1982. Nitrite reduction to nitrous oxide by propionibacteria: Detoxification Mechanism. Arch. Microbiol. **133:**126-130.

Kemp, A., J. Geurink, R. Haalstra, and A. Malestein. 1976. Nitrate poisoning in cattle. 1. Discolouration of the vaginal mucous membrane as aid in the prevention of nitrate poisoning in cattle. Stikstof. **19**:40-48.

Kemp, A., J. Geurink, Haalstra,and A. Malestein. 1977. Nitrate poisoning in cattle. 2. Changes in nitrite in rumen fluid and methemoglobin formation after high nitrate intake. Neth. J. agric. Sci. **25**:51-62.

Kemp, A., J. Geurink, A. Malestein, and A. van't Klooster. 1978. Grassland production and nitrate poisoning in cattle. Proc. 7th gen. Meet. Eur. Grassld. Fedn. (Gent).

Lyon, W., and B. Glatz. 1991. Partial purification and characterization of a bacteriocin produced by *Propionibacterium theonii*. Appl. Environ. Microbiol. **57**:701-706.

MacGregor, C., C. Schnaitman, D. Normansell, and M. Hodgins. 1974. Purification and properties of nitrate reductase from *Escherichia coli* K12. J. Biol. Chem. **249**:5321-5327.

MacGregor, C. 1975a. Solubilization of *Escherichia coli* nitrate reductase by a membrane-bound protease. J. Bacteriol. **121**:1102-1110.

MacGregor, C. 1975b. Anaerobic cytochrome b1 in *Escherichia coli*: association with and regulation of nitrate reductase. J. Bacteriol. **121**:1111-1116

MacGregor, C. 1975c. Synthesis of nitrate reductase components in chlorate-resistant mutants of *Escherichia coli*. J. Bacteriol. **121**:1117-1121.

MacGregor, C., and A. Christopher. 1978. Asymmetric distribution of nitrate reductase subunits in the cytoplasmic membrane of *E. coli*: evidence derived from surface labeling studies with transglutaminase. Arch. Biochem. Biophys. **185**:204-213.

MacGregor, C., and G. McElhaney. 1981. New mechanism for post-translational processing during assembly of a cytoplamic membrane protein? J. Bacteriol. **148**:551-558.

Malestein, A., J. Geurink, G. Schuyt, A. Schotman, A. Kemp,and A. van't Klooster. 1980. Nitrate poisoning in cattle. 4. The effect of nitrite dosing during parturition on the oxygen capacity of maternal blood and the oxygen suppley to the unborn calf. Vet. Quart. **2**:149-159.

Mantere-Alhonen, S. 1982. Die Propionibakterien der Molkereiindustrie als Darmkanalmikroben. Meijeritieteellinen Aikakauskirja. **XL** (1) 95 S.

Mantere-Alhonen, S. 1983. On the survival of a *Propionibacterium freudenreichii*-culture during 'in vitro' gastric digestion. Meijeritieteellinen Aikakauskirja. **XLI**:19-23.

Mantere-Alhonen, S., and E. Makinen. 1987. A new type of sour milk with propionibacteria. Meijeritieteellinen Aikakauskirja. XLV:49-61.

Mantere-Alhonen, S., and H. Mllymaki. 1985. A substrate for the production of propionibacteriamass. Kemia-Kemi short communications, n:o 11.

Marwha, S., J. Kennedy, and R. Sethi. 1983. Vitamin B_{12} production from whey and simulation of optimal cultural conditions. Process. Biochem. **18**:24-27.

McPherson, M., A. Baron, D. Pappin, and J. Wootton. 1984. Respiratory nitrate reductase of *Escherichia coli*. Sequence identification of the large subunit gene. FEBS Lett. **177**:260-264.

Nabukhotnyi, T., S. Cherevko, G. Sanigullina, and A. Grushko. 1985. Use of adapted propioni-acidophilic "Malyutka" and "Malyush" formulas in complex treatment of acute gastrointestinal diseases of infants. Ref. Dairy Sci. Abstr. 1985, **47**:2122.

Nohno, T., S. Noji, S. Taniguchi, and T. Saito. 1989. The *narX* and *narL* genes encoding the nitrate-sensing regulators of *Escherichia coli* are homologous to a family of prokaryotic two-component regulatory genes. Nucleic Acids Res. **17**:2947-2957.

Noji, S., T. Nohno, T. Saito, and S. Taniguchi. 1989. The *narK* gene product participates in nitrate transport induced in *Escherichia coli* nitrate-respiring cells. FEBS. **252**:139-143.

Park, H., G. Reinbold, and E. Hammond. 1967. Role of propionibacteria in split defect of Swiss cheese. J. Dairy Sci. **50**:820-823.

Perez-Mendoza, J. and F. Garcia Hernandez. 1983. Fermentation of a waste-product from the industrial processing of the lime (Citrus aurantifolia swingle) for vitamin B_{12} production. I. Culture conditions for *Propionibacterium shermanii* and effect of 5,6-dimethylbenzimidazole as precursor. Biotechnol. Lett. **5**:259-264.

Perlman, D. 1978. p.303-326. In: Rose, A. H. (ed.) Economic microbiology, vol. 2., Primary products of metabolism. Academic Press Inc., London.

Playne, M. 1985. p.731-759. Propionic and butyric acids. In: Moo-Young, M. (ed.) Comprehensive biotechnology. Pergamon Press, Oxford.

Rabin, R., L. Collins,and V. Stewart. 1992. In vivo requirement of intergration host factor for nar (nitate reductase) operon expression in *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA. **89**:8701-8705.

Rabin, R., and V. Stewart. 1992. Either of two functionally redundant sensor proteins, *narX* and *narQ* is sufficient for nitrate regulation in *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA. **89**:8419-8423.

Rabin, R. ,and V. Stewart. 1993. Dual response regulators (*narL* and *narP*) interact with dual sensors (*narX* and *narQ*) to control nitrate- and nitrite- regulated gene expression in *Escherichia coli* K-12. J. Bacteriol. **175**:3259-3268.

Radcliffe, B. and D. Nicholas. 1970. Some properties of a nitrate reductase from *Pseudomonas denitrificans*. Biochim. Biophys. Acta. **205**:273-287.

Raeker, M., C., Bern, L. Johnson and B. Glatz. 1992. Preservation of high-moisture maize by various propionate treatments. Cereal Chem. **69**:66-69.

Rehberger, T., C. Hibberd, and J. Swartzlander. 1993. Identification and application of a propionibacteria strain for nitrate and nitrite reduction in the rumen, p.E1-E15. *In* G. Highfill (ed.), Management of high nitrate forages for beef and dairy cattle. Okla. Coop. Ext. Serv., Enid.

Riet, J. van't (1970). Ph.D. Thesis: Free University, Amsterdam.

Riet, J. van't, A. Stouthamer, and R. Planta. 1968. Regulation of nitrate assimilation and nitrate respiration in *Aerobacter aerogenes*. J. Bacteriol. **96**:1455-1464.

Riet, J. van't, and R. Planta. 1969. Purification and some properties of the membrane-bound respiratory nitrate reductase of *Aerobacter aerogenes*. FEBS. **5**:249-252.

Riet, J. van't, J. van Ed, R. Wever, B. van Gelder, and R. Planta. 1975. Characterization of the respiratory nitrate reductase of *Klebsiella aerogenes* as a molybdenum-containing iron-sulfur enzyme. Biochim. Biophys. Acta. **405**:306-317.

Ronson, C., B. Nixon, and F. Ausubel. 1987. Conserved domains in bacterial regulatory proteins that respond to environmental stimuli. Cell **49**:579-581.

Ruiz-Herrera, J., and J. DeMoss. 1969. Nitrate reductase complex of *Escherichia coli* K-12: isolation and characterization of mutants unable to reduce nitrate. J. Bacteriol. **97**:1291-1297.

Ruiz-Herrera, J., and J. DeMoss. 1969. Nitrate reductase complex of *Escherichia coli* K12: participation of specific formate dehydrogenase and cytochrome b1 component in nitrate reduction. J. Bacteriol. **99**:720-729.

Sanigullina, F. I., and W. Pawliuk. 1984. Eine experimentelle beurteilung der biologishchen wertigkeit der Propionsaure Acidophilus-Nahrmittel "Malyutka" und "Malyush". Ref. Mi. 1985, 585.

Schulp, J. and A. Stouthamer. 1970. The influence of oxygen, glucose and nitrate upon the formation of nitrate reductase and the respiratory system in *Bacillus licheniformis*. J. Gen. Microbiol. **64**:195-203.

Schulp, J. 1972. Ph.D. Thesis: Free University, Amsterdam.

Shaw, D., and J. Guest. 1983. Homology between CAP and Fnr, a regulator of anaerobic respiration in *Escherichia coli*. J. Mol. Biol. **166**:241-247.

Showe, M., and J. DeMoss. 1968. Localization and regulation of synthesis of nitrate reductase in *Escherichia coli*. J. Bacteriol. **95**:1305-1313.

Sidorchuk, L., and V. Bondarenko. 1984. Selection of a biologically active mutant of *Propionibacterium shermanii* and the possibility of its use in complex therapy of enteral dysbacteriosis. J. Hyg. Epidemiol. Microbiol. Immunol. **28**:331-333.

Sidorchuk, L., V. Bondareko, B. Patraty, S. Cherevko, and T. Nabukhotny. 1984. Efficiency of biopreparation from *Propionibacterium shermanii* in prophylaxis of dysbacteriosis in salmonellosis and shigellosis treatment. Mikrobiol. ZH. **46**:43-46.

Simon, J., J. Sund, M. Wright, A. Winter, and F. Douglas. 1958. Pathological changes associated with the lowland abortion syndrome in Wisconsin. J. Am. Vet. Med. Assoc. **132**:164.

Simoni, R., and M. Schallenberger. 1972. Coupling of energy to active transport of amino acids in *Escherichia coli*. Proc. Natl. Acad. Sci. USA. **69**:2663-2667.

Sinclair, P., and D. White. 1970. Effect of nitrate, fumarate, and oxygen on the formation of the membrane-bound electron transport system of *Haemophilus parainfluenzae*. J. Bacteriol. **101**:365-372.

Sodergren, E., and J. DeMoss. 1988. *narI* region of the *Escherichia coli* nitrate reductase (*nar*) operon contains two genes. J. Bacteriol. **170**:1721-1729.

Somkuti, G., and T. Johnson. 1990. Cholesterol uptake by *Propionibacterium freudenreichii*. Curr. Microbiol. **20**:305-309.

Sone, N. (1972) The redox reactions in propionic acid fermentations. Occurrence and nature of an electron transfer system in *Propionibacterium arabinosum*. J. Biochem. **71**:931-940.

Spiro, S. and J. Guest. (1990). FNR and its role in oxygen-regulated gene expression in *Escherichia coli*. FEMS Microbiol. Lett. **75**:399-428.

Stewart, V. 1982. Requirement of Fnr and NarL functions for nitrate reductase expression in *Escherichia coli* K-12. J. Bacteriol. **151**:1320-1325.

Stewart, V. 1988. Nitrate respiration in relation faculative metabolism in *Enterobacteriaceae*. Microbiol. Rev. **52**:190-232.

Stewart, V., and C. MacGregor. 1982. Nitrate reductase in *Escherichia coli* K12: involvement of *chlC*, *chlE*, and *chlG* loci. J. Bacteriol. **151**:788-799.

Stewart, V., and J. Parales, Jr. 1988. Identification and expression of genes *narL* and *narX* of the *nar* (nitrate reductase) locus in *Escherichia coli* K-12. J. Bacteriol. **170**:1589-1597.

Stewart, V., J. Parales, Jr., and S. Merkel. 1989. Structure of genes *nar*L and *nar*X of the *nar* (nitrate reductase) locus in *Escherichia coli* K-12. J. Bacteriol. **171**:2229-2234.

Stouthamer, A. 1967. Mutant strains of *Aerobacter aerogenes* which require both methionine and lysine for aerobic growth. J. Gen. Microbiol. **46**:389-398.

Stouthamer, A. 1976. Biochemistry and genetics of nitrate reductase in bacteria. Adv. Microb. Physiol. **14**:315-375.

Tabib, Z., W. Hagler, and P. Hamilton. 1982. Comparison of physiological parameters useful for assessment of activity of antifungal agents in feed and ingredients. J. Food Prot. **45**:1030-1037.

Tomes, N. 1991. Bacterial treatment to preserve silage. US Patent 4,981,705.

Tuikov, R. M. Khristova, K. Rizanvov, and I. Vladimirov. 1980. Early weaning of calves using a milk additive and a preparation of freeze-dried bacteria. Dairy Sci. Abstr. 1982, 45 (4):1689.

Vladimirov, I., K. Rizanvov, A. Vasilev, and P. Kenov. 1977. Tests on replacement of antibiotics in sucking calf feeding by a freeze-dried preparation of bacteria. Dairy Sci. Abstr. 1978. **40** (4):2118.

Weiner, J., D. MacIsaac, R. Bishop, and P. Bilous. 1988. Purification and properties of *Escherichia coli* dimethylsulfoxide reductase, an iron-sulfer molybdoenzyme with broad substrate specificity. J. Bacteriol. **170**:1505-1511.

Wimpenny, J., and J. Cole. 1967. The regulation of metabolism in faculative bacteria. III. The effect of nitrate. Biochim. Biophys. Acta. **148**:233-242..

Wimpenny, J. 1969. Oxygen and carbon dioxide as regulators of microbial growth and metabolism. Symposium of the Society for General Microbiology. **19**:161-197.

Woolford, M. 1975. The significance of *Propionibacterium* species and *Micrococcus lactilyticus* to the ensiling process. J. Appl. Bacteriol. **39**:301-306.

Yongsmith, B., and P. Apiraktivongse. 1983. Vitamin B_{12} production from soybean curd whey with *Propionibacterium freudenreichii*. J. Ferment. Technol. **61**:105-107.

CHAPTER III

GENETIC ANALYSIS OF THE NITRATE REDUCTASE GENE OF *PROPIONIBACTERIUM*

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ABSTRACT

PCR amplification and Southern hyridizations were performed to characterize the nitrate reductase gene from chromosomal DNA of 10 *Propionibacterium* strains. Various sized fragments were produced using a primer set based on regions of homology in *E. coli* molybdoenzymes. PCR amplification of *Propionibacteria acidipropionici* strain P5 produced the same size fragment (1.8 kb) as the product amplified from the *Escherichia coli* nitrate reductase gene, *narG*. Southern hybridizations indicated that the P5 1.8 kb PCR fragment was homologous to the 1.8 kb PCR fragment of *E. coli*. Southern hybridization also revealed that P5 and two other strains, *Propionibacteria freudenreichii* strains P93 and P103, produced 1.35 kb fragments that were homologous to the *E. coli* 1.8 kb PCR product. Restriction endonuclease digestions and DNA sequencing data indicate that the 1.8 kb PCR product from P5 is identical to a 1.8 kb region of the *narG* gene in *E. coli*.

INTRODUCTION

Propionibacteria are industrially important organisms used primarily in the dairy industry as starter cultures for the production of Swiss cheese (Biede and Hammond, 1979; Langsgrud and Reinbold, 1973a; Langsgrud and Reinbold, 1973b). Other applications of these bacteria have been described which include the use as a human probiotic (Sidorchuk and Bondarko, 1984; Sidorchuk et al., 1984; Korneya, 1982; Nabukhotnyi, 1985; Mantere-Alhonen, 1987; Sanigullina, 1985; Cerna, 1984; Somkuti and Johnson, 1990), silage inoculant (Woolford, 1975; Flores-Galarza et al., 1985; Raeker et al., 1992; Dawson, et al., 1991; Tomes, 1991), anti-microbial agent (Lyon and Glatz, 1991; Grinstead and Barefoot, 1992) and in the production of vitamin B_{12}, and propionic acid (Perlman, 1978; Playne, 1985). Additionally propionibacteria have been used as direct fed microbials for animals (Vladimirov, et al., 1978; Tuikov, 1982; Mantre-Althonen, 1982;1983;1985; Antipov, 1980). A recent study investigated the use of propionibacteria as a direct fed microbial to reduce the toxic effects of nitrates in cattle (Rehberger et al., 1993).

Presently there is limited research information about the denitritification enzymes of propionibacteria, and no genetic analyses have been performed. A general understanding of the genes encoding these enzymes may provide the potential of improved direct fed microbial strains through genetic engineering.

In this study we characterized the propionibacteria nitrate reductase gene, using primers derived from conserved sequences in the nitrate reductase of *Escherichia coli.* Southern hybridizations were performed to demonstrate DNA-DNA homologies between the propionibacteria strains PCR products and that of the PCR product amplified from the

Escherichia coli nitrate reductase *narG* gene. Additional comparisons between the 1.8 kb product from *Propionibacteria acidipropionici* strain P5 and the predicted 1.8 kb product from *E. coli* PCR were performed by restriction endonuclease digestions and DNA sequencing.

Materials and Methods

Bacterial strains and culture conditions. All *Propionibacterium* strains were obtained from the culture collection of the Department of Animal Science at Oklahoma State University. The *Propionibacterium* strains used in this study are listed in table 1. Strains were grown in sodium lactate broth (NLB) at 32 °C and maintained on (NLB + 1.5% agar)(Hofherr and Glatz,1983). *Propionibacterium* strain P5 was grown in NLB + 1% glycine prior to the isolation of intact chromosomal DNA.

Chromosomal DNA isolation and purification. Chromosomal DNA was isolated from propionibacteria using a preparative scale DNA isolation procedure (Rehberger, Ph.D. thesis). Chromosomal DNA was purified by CsCl-ethidium bromide density gradient centrifugation (Maniatis et al., 1982). Gradient purified DNA samples were extracted using isopropanol saturated with 5M NaCl followed by desalting and concentration in 10 mM Tris-HCl-1 mM EDTA pH 7.5, using a Centricon-30 microconcentrator according to the instructions of the manufacturer (Amicon Corp., Danvers, Mass.).

Preparation of intact genomic DNA in agarose beads. Intact genomic DNA from *Propionibacteria acidipropionici* strain P5 was isolated from cells embedded in agarose beads for use in pulse-field electrophoresis by a modification of the method of Kauc et al., (1989) as performed by Rehberger (1993). Cultures were grown to mid-log stage, harvested by centrifugation (9,000 g for 10 min), and resuspended using ET buffer (50 mM EDTA, 1 mM Tris-HCl, pH. 8.0) to an OD of 20. The cell suspension was warmed to 45 ° C and mixed with an equal volumeof 1% low-melting-point agarose.

Table 1. *Propionibacterium* strains used in study

OSU #	Species and strain designation		Nitrate reduction	Denitrification
P5	*P. acidipropionici*	E214	+	+
P58	*P. acidipropionici*	ATCC 4875	+	+
P7	*P. freudenreichii*	52	+	-
P22	*P. freudenreichii*	123	+	+
P93	*P. freudenreichii*	CNRZ91	+	+
P103	*P. freudenreichii*	5932	+	-
P113	*P. freudenreichii*	F32	+	-
P63	*P. jensenii*	PJ54	+	+
P9	*P. jensenii*	129	+	-
P42	*P. jensenii*	10	+	+
P63	*P. jensenii*	PJ54	+	+

Two volumes of paraffin oil (prewarmed 45 °C) was added to the cell-agarose mixture and throughly mixed by repeated inversion of the tube. The emulsion was immediately poured into cold ET buffer with constant slow stirring to encapsulate the cells in agarose beads. After incubation on ice for 10 min the paraffin oil was removed from the agarose beads by centrifugation (4,000 g for 10 min). The beads were resuspended in 10 ml of 10X ET buffer containing 20 mg/ml of lysozyme and were incubated at 32 °C for 2 h to digest the cell wall material. After incubation, the beads were harvested by centrifugation and resuspended in 10 ml of lysis buffer (10X ET buffer containing 100 μg/ml of proteinase K and 1% [w/v] Sarkosyl). The beads were incubated at 55 °C for 5-7 h. After cellular lysis, the beads were harvested by centrifugation (4,000 g for 10 min), resuspended in 10 ml of 1 mM phenylmethylsulfonylfluoride, and incubated at room temperature for 2 h with gentle agitation. The beads containing the purified DNA were washed three times in TE buffer (10 mM Tris-HCl, 1mM Na_2-EDTA, pH. 7.5), resuspended in 10 ml of TE buffer, and stored at 4 °C until restriction endonuclease digestion.

Polymerase Chain Reaction. Two particularly well conserved domains within the NarG encoded polypeptide of the nitrate reductase and other molybdoenzymes of *Escherichia coli* K12 were selected as the basis for primer synthesis. The DNA sequence of the coding strand primer is 5' TCCTGGCAGGAGGTG 3' (161-Ser-Trp-Asp-Glu-Val-165). The complementary strand primer 5' GACATCATTTTGCCG 3' corresponds to consensus sequence (776-Asp-Ile-Ile-Leu-Pro-780). Non-degenerate primers (exact sequences from K12) were used in the PCR reactions to avoid the amplification of numerous unrelated DNA sequences.

Optimization for PCR amplified fragments was performed varying different concentrations of DNA, $MgCl_2$, dNTP, and primers. Annealing temperatures were also varied. Initial amplification optimizations were carried out using DNA from *E. coli* strain B, (Sigma Chemical Co., St. Louis, MO). PCR conditions allowing the best amplification

of *E. coli* strain B chromosomal DNA included an initial denaturation step at 95 °C for 2 min, an annealing step at 58 °C for 1 min and an extension step at 72 °C for 3 min, followed by 29 cycles (94 °C for 1 min, 58 °C for 1 min, then 72 °C for 2 min) and a final extension at 72 °C for 5 min. PCR reaction mixtures contained: 5 µl of 10X reaction buffer (50 mM KCl, 10 mM Tris-HCl), 2.7 mM $MgCl_2$ (Boehringer Mannheim, Indianapolis, IN), 300 µM of dNTP (Boehringer Mannheim), 30 nM of primers, 1.75 units of Taq DNA polymerase (Boehringer Mannheim), 10 ng of chromosomal DNA and ddH_20 to a volume of 50 µl. Reaction mixtures were topped with 50 µl of mineral oil and amplified in a Perkin Elmer thermal cycler model number 480 (Perkin Elmer, Norwalk, Ct.). *E. coli* was used as a positive control for every PCR reaction. A more narrow range of conditions was examined for the optimization of PCR amplification using propionibacteria DNA (tables 2-5). PCR conditions allowing the best amplification of all propionibacteria strains included an initial denaturation step at 95 °C for 2 min, an annealing step at 50 °C for 1 min, and a extension step at 72 °C for 3 min, followed by 29 cycles (94 °C for 1 min, 50 °C for 1 min, then 72 °C for 2 min) and a final extension at 72 °C for 5 min. PCR reaction mixtures contained as follows: 5 µl of 10X reaction buffer (50 mM KCl, 10 mM Tris-HCl), 1.5 mM $MgCl_2$ (Promega Corp., Madison, Wisc.), 300 µM of dNTP (Boehringer Manheim), 30 nM of primers, 1.75 units of Taq DNA polymerase (Boehringer Manheim), 10 ng of chromosomal DNA and ddH_20 to a volume of 50 microliters.

Agarose gel electrophoresis. PCR products were separated using agarose gel electrophoresis. Reaction products were separated in 1% agarose gels run in Tris-borate buffer for 2 hours at 100 V. Lambda phage digested with *Pst* I was used as a molecular size standard. Gels were stained in distilled water containing 5 µg/ml of ethidium bromide for one hour, viewed on an UV transilluminator (Foto UV 300; Fotodyne Inc., New Berlin, Wis.), and photographed through 23A and 2B Wratten gel filters with a Polaroid MP4 camera (film type 55) (Meyers et al., 1976).

Table 2. PCR optimization of *E. coli* strain B chromosomal DNA (quantities and temperatures attempted)

Variables	Experimental values
300 mM MgCl	2.5, 5.0, 6.0, 7.0, 7.5, 10.0 µl
200 mM dNTP	0.75, 1.0, 1.5 µl
5 µM primers	2.0, 3.0, 4.0, 6.0 µl
DNA (ng)	1.0, 2.0, 10.0, 20.0
Temperatures °C	50.0, 55.0, 58.0

Table 3. PCR optimization of *Propionibacterium* strain P58 chromosomal DNA (quantities and temperatures attempted).

Variables	Experimental values
300 mM MgCl	2.5, 3.5, 4.0, 4.5, 5.0, 6.0, 7.0, 9.0 µl
200 mM dNTP	0.75, 1.0, 1.5, 2.0, 2.5 µl
5 µM primers	2.0, 2.5, 3.0, 3.5, 4.0, 6.0 µl
DNA (ng)	10.0
Temperatures °C	47.0, 50.0, 55.0

Table 4. PCR optimization of *Propionibacterium* strains P5, P22, P93. and P103 chromosomal DNA (quantities and temperatures attempted).

Variables	Experimental values
300 mM MgCl	3.5, 4.0, 4.5, 5.0, 6.0, 7.0, 9.0 µl
200 mM dNTP	1.5, 2.0, 2.5 µl
5 µM primers	3.0 µl
DNA (ng)	10.0
Temperatures °C	47.0, 50.0, 55.0

Table 5. Serial PCR optimization of *Propionibacterium* strain P5 DNA (quantities and temperatures attempted).

Variables	Experimental values
300 mM MgCl	5.0, 7.0, 9.0 µl
200 mM dNTP	1.5, 2.0, 2.5 µl
5 µM primers	3.0 µl
Initial reaction DNA	1.0, 5.0, 10.0 µl
Temperatures °C	50.0, 55.0

Extraction of PCR fragments for use as probes for Southern blotting. Amplified PCR products were applied to a preparative agarose gel, run, and stained in ethidium bromide. The desired putative partial nitrate reductase encoding fragment from chosen strains was cut out and the DNA eluted using the Elutrap DNA extraction kit according to instructions of the manufacturer (Schleicher & Schuell, Inc., Keene, NH).

Restriction endonuclease digestion of encapsulated DNA and transverse alternating field gel electrophoresis. Ninety microliters of agarose beads per digest were equilibrated in 1X restriction endonuclease digestion buffer at 4°C for 1 h. After equilibration 10-15 units of either *AsnI, Hpa I, SnaB I, Ssp I, Vsp I,* or *Xba I* were added to prepared chromosomal DNA containing sample beads and digested for 6 h at the recommended temperature. After digestion, agarose beads were melted at 65°C for 10 min and loaded into the wells of the agarose gel. The melted beads were allowed to solidify, and the wells were capped with molten agarose before the gel was placed in the TAFE II apparatus (Beckman Instruments, Inc. Palo Alto, CA). DNA fragments were separated on 1% agarose gels in 20 mM Tris, 0.5 M EDTA, pH 8.2, at 10-15°C in a TAFE II geneline pulse field system using a program consisting of separate, consecutive stages of different pulse times, all at a constant field strength of 350 mA. The program consisted of six 3-h stages with pulse times increasing from 7 to 17s in 2s increments. An additional set of beads was run in a CHEF mapper (BioRad, Hercules, CA) using the computer settings to optimally separate DNA ranging from 15 to 300 Kb.

Southern Hybridization. PCR amplified fragments were transferred to a nylon membrane (Micron Separations Inc., Westboro, MA) using the Southern blotting technique (Southern, 1975). Labeled Eco T141 digested lambda DNA was used as the molecular size standard (Amersham Corp., Arlington Heights, IL). *Escherichia coli* strain B, and *Propionibacterium* stains P5 and P58 were used individually to reprobe the first Southern blot. An ECL random prime labeling and detection system was used as described by the manufacturer (Amersham Corp., Arlington Heights, IL). The *E. coli* B

PCR amplified fragment was digested with *Xho* I and *Bgl* II and separated using agarose gel electrophoresis to remove PCR primers. Bal 31 digestion was used to remove the primers of the P5 and P58 PCR fragments used for probes. The remaining enzyme digested fragments from each of the three strains were then labeled using the ECL labeling system. Prehybridization, hybridization, posthybridization washes, filter blocking, and detection of homologous sequences was performed as described by the manufacturer of the ECL system.

Restriction endonuclease digestion of P5 and *E. coli* 1.4 Kb PCR products. Twenty microliters of the PCR reactions designed to amplify only an internal 1.4 Kb fragment from the 1.8 kb fragment from P5 and *E. coli* were used to compare digestion patterns between the two products. Ten units of either Alu I, Bstn I, BstY I, Fok I, Hae III, Hinc II, MspI, Sau3 AI, TaqI were used to restrict the fragments. Each sample was electrophoresed in a 4% nusieve:agarose (1:1 ratio) mixture gel and run 1-2 h at 75 V.

Sequencing and analysis of P5 nitrate reductase PCR product. DNA for sequencing was purified and concentrated using preparative agarose gel electrophoresis and a Gene Clean II DNA purification kit (Biolab 101) to a concentration of .2 μg/μl. Extracted DNA was then sequenced using the dideoxy chain termination method (Sanger et al., 1977) using a Sequenase version 2.0 DNA Sequencing Kit (United States Biochemical, Cleveland, OH.). PCR product DNA was heat denatured by boiling for 5 min and then quick frozen in a 50%/50% ethanol/methanol mixture at -80 °C. The instructions of the Sequenase 2.0 kit were followed for the remainder of the reaction. This procedure was used to sequence the first 225 bases from each end of the fragment. The remainder of the product was sequenced by Sequtech Corp. (Mountainview, CA). DNA sequences were analyzed using MacVector and AssemblyLIGN software (International Biotechnologies, Inc., New Haven, CT).

Results

PCR amplified fragments. PCR amplification of chromosomal DNA from *E. coli* strain B using primers based on the *E. coli* nitrate reductase sequence yielded a fragment of the predicted molecular weight (1.87 kb). Bgl II and Xho I restriction endonuclease digests of the 1.87 kb PCR product confirmed that this amplified fragment was from the internal region of the *narG* gene.

PCR amplification of chromosomal DNA from propionibacteria strain P58 consistently produced a fragment that was estimated to be 1.49 kb. Strain P5 and the remaining propionibacteria strains produced no DNA fragments visible in agarose gels after a single amplification reaction. A second PCR amplification or sequential PCR process was necessary to produce DNA fragments visible on agarose gels. In this process, 1 µl from the first PCR reaction was used as template for the second PCR amplification. Using this technique, chromosomal DNA from propionibacteria strain P5 yielded a 1.36 kb fragment and a 1.8 kb fragment, similar in size to the *E. coli* PCR product. All other propionibacteria strains produced a variety of fragment sizes. Most strains produced more than one visible fragment, often with a single fragment of greater intensity and numerous less intense fragments. No noticeable pattern in the PCR amplified products was observed between strains of the same species.

Southern hybridizations. Southern hybridizations were performed using the *E. coli* 1.87 kb fragment as a probe following enzyme digestion to remove the primer ends. Target DNA included PCR products from all propionibacteria strains and *E. coli*. The 1.87 kb probe hybridized to the 1.83 kb fragment from propionibacteria strain P5, the 1.36 kb fragments from propionibacteria strains P5, P93 and P103 and as expected, the 1.87 kb fragment from *E. coli* (fig. 1). The 1.36 kb PCR products from strains P93 and P103

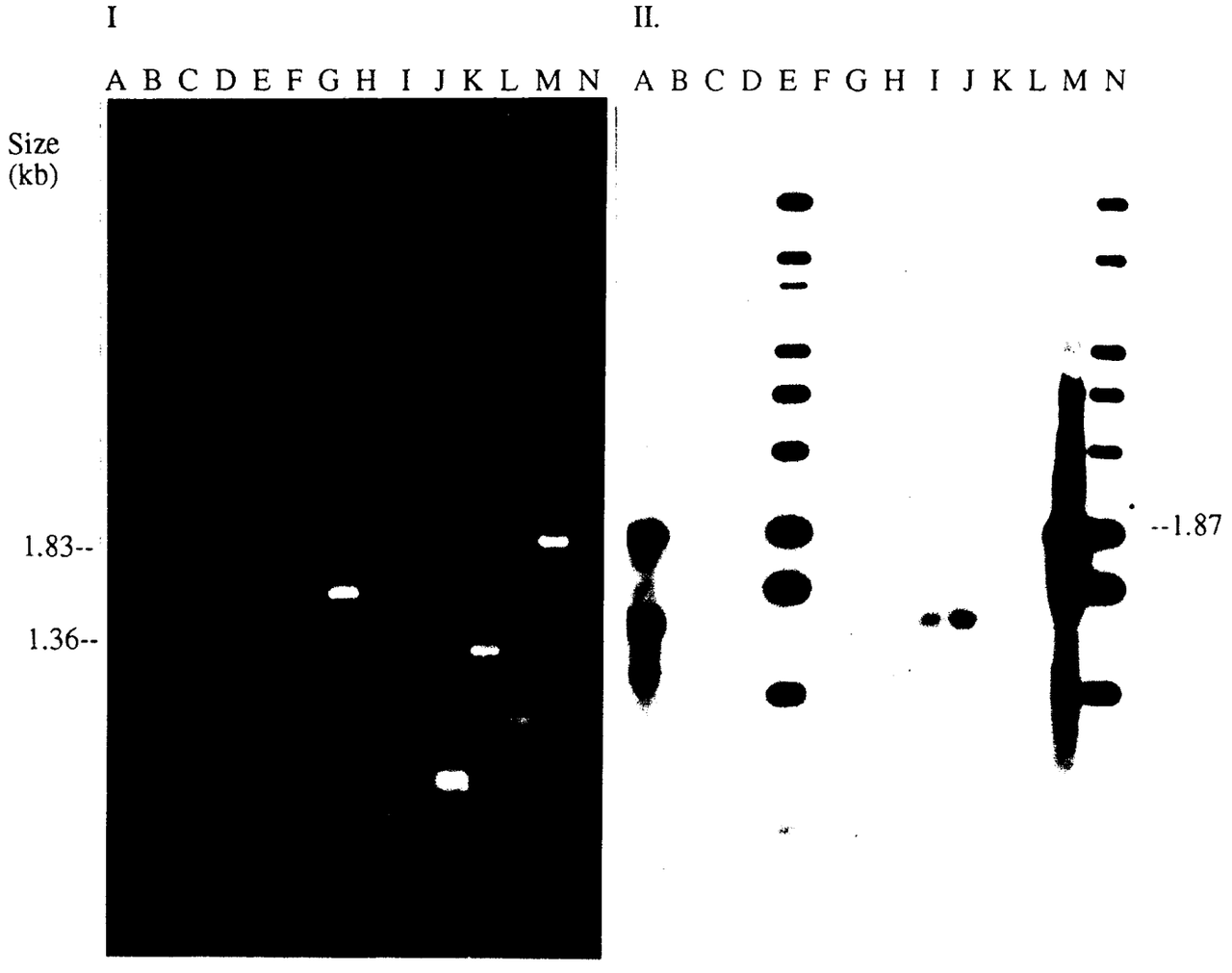

I
A B C D E F G H I J K L M N
Size
(kb)
1.83--
1.36--
II.
A B C D E F G H I J K L M N
--1.87

Figure 1. Hybidization of HRP-labeled *Escherichia coli* strain B 1.87 Kb PCR product DNA to PCR products of various strains of *Propionibacterium* chromosomal DNA. (I.) Agarose gel electrophoresis of PCR products of strains of *Propionibacterium* and *Escherichia coli* strain B chromosomal DNA. Lanes; A, P5 PCR product DNA; B, P7 PCR product DNA; C, P9 PCR product DNA; D, P22 PCR product DNA; E, Eco T141 digest of lambda DNA molecular weight marker; F, P42 PCR product DNA; G, P58 PCR product DNA; H, P63 PCR product DNA; I, P93 PCR product DNA; J, P103 PCR product DNA; K, P104 PCR product DNA; L, P113 PCR product DNA; M, *E. coli* B PCR product DNA; N, EcoT141 digest of lambda DNA molecular weight marker. (II). Hybridization of the *Escherichia coli* strain B 1.87 Kb probe to a nitrocellulose filter containing PCR product DNA shown in Panel I.

were at such low concentrations that they were not visible in the agarose gels. No other PCR products were detected with the 1.87 kb probe.

When the 1.5 kb PCR fragment from strain P58 was used as a probe (primer ends removed), a 1.0 kb fragment from strain P58 was detected along with the 1.5 kb fragment (fig.2). The 1.0 kb fragment was not visible in the agarose gel. No other PCR products were detected with the 1.5 kb probe.

Hybridization with the primer deleted 1.83 kb PCR fragment from strain P5 is shown in figure 3. PCR fragments detected by the 1.87 kb *E. coli* probe were also detected with this probe. In addition, the P5 1.83 kb probe hybridized to two fragments not detected by the 1.87 kb *E. coli* probe, a 1.36 kb fragment from strain P42 and a 1.83 kb fragment form P93. However, both of these signals were faint and may have been detected only because of the longer exposure time. A summary of the hybridizing probes is listed in table 6.

PCR amplification using a second primer pair and restriction digestion analysis of the products. Based on initial sequence information form the 1.83 kb fragment from strain P5, a second primer pair was synthesized and used to amplify an internal region of the 1.83 kb fragment. Amplification of P5 DNA with this primer set yielded a 1.4 kb fragment in a single PCR reaction. Amplification of *E. coli* DNA also yielded a 1.4 kb fragment. In order to determine if these fragments were homologous in structure, restriction digests of the 1.4 kb fragments were performed using a series of enzymes selected based on analyses of the published *E. coli* sequence information. Restriction fragments patterns of the 1.4 kb fragment from P5 and *E. coli* were identical for all enzymes examined (fig. 4) indicating a high degree of similarity.

Sequence comparison of the P5 1.8 kb PCR product with the *E. coli* 1.8 kb PCR *narG* fragment. To determine the degree of sequence homology between the 1.8 kb fragments of P5 and *E. coli*, the 1.8 kb PCR fragment from P5 was sequenced (Sequencing strategy, fig. 5). Comparison of the nucleotide sequence showed that the 1.8

Figure 2. Hybidization of HRP-labeled *Propionibacterium* strain P58 1.5 Kb PCR product DNA to PCR products of various strains of *Propionibacterium* chromosomal DNA. (I.) Agarose gel electrophoresis of PCR products of strains of *Propionibacterium* and *Escherichia coli* strain B chromosomal DNA. Lanes; A, P5 PCR product DNA; B, P7 PCR product DNA; C, P9 PCR product DNA; D, P22 PCR product DNA; E, EcoT141 digest of lambda DNA molecular weight marker; F, P42 PCR product DNA; G, P58 PCR product DNA; H, P63 PCR product DNA; I, P93 PCR product DNA; J, P103 PCR product DNA; K, P104 PCR product DNA; L, P113 PCR product DNA; M, *E. coli* B PCR product DNA; N, EcoT141 digest of lambda DNA molecular weight marker. (II). Hybridization of the *Propionibacterium* strain P58 1.49 Kb probe to a nitrocellulose filter containing PCR product DNA shown in Panel I.

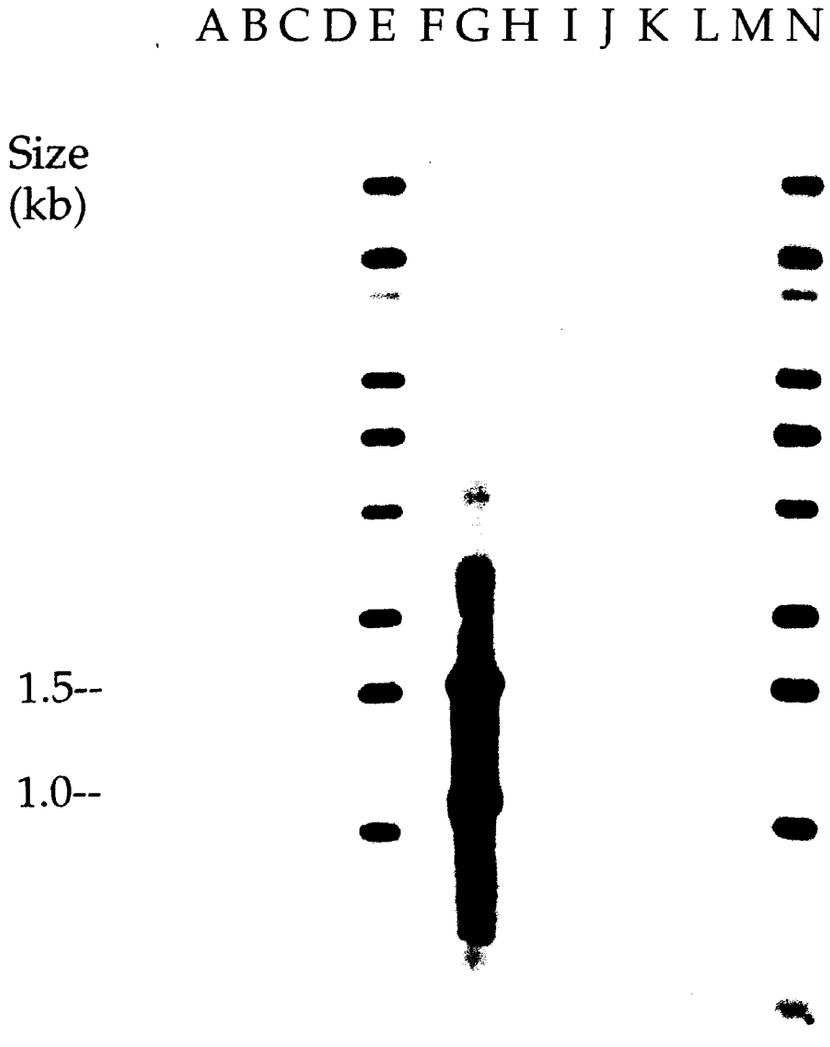

A B C D E F G H I J K L M N
Size
(kb)
1.5--
1.0--

Figure 3. Hybidization of HRP-labeled *Propionibacteria acidi-propionici* strain P5 1.83 Kb PCR product DNA to PCR products of various strains of *Propionibacterium* chromosomal DNA. (I.) Agarose gel electrophoresis of PCR products of strains of *Propionibacterium* and *Escherichia coli* strain B chromosomal DNA. Lanes; All lanes correspond to that of figure 1.

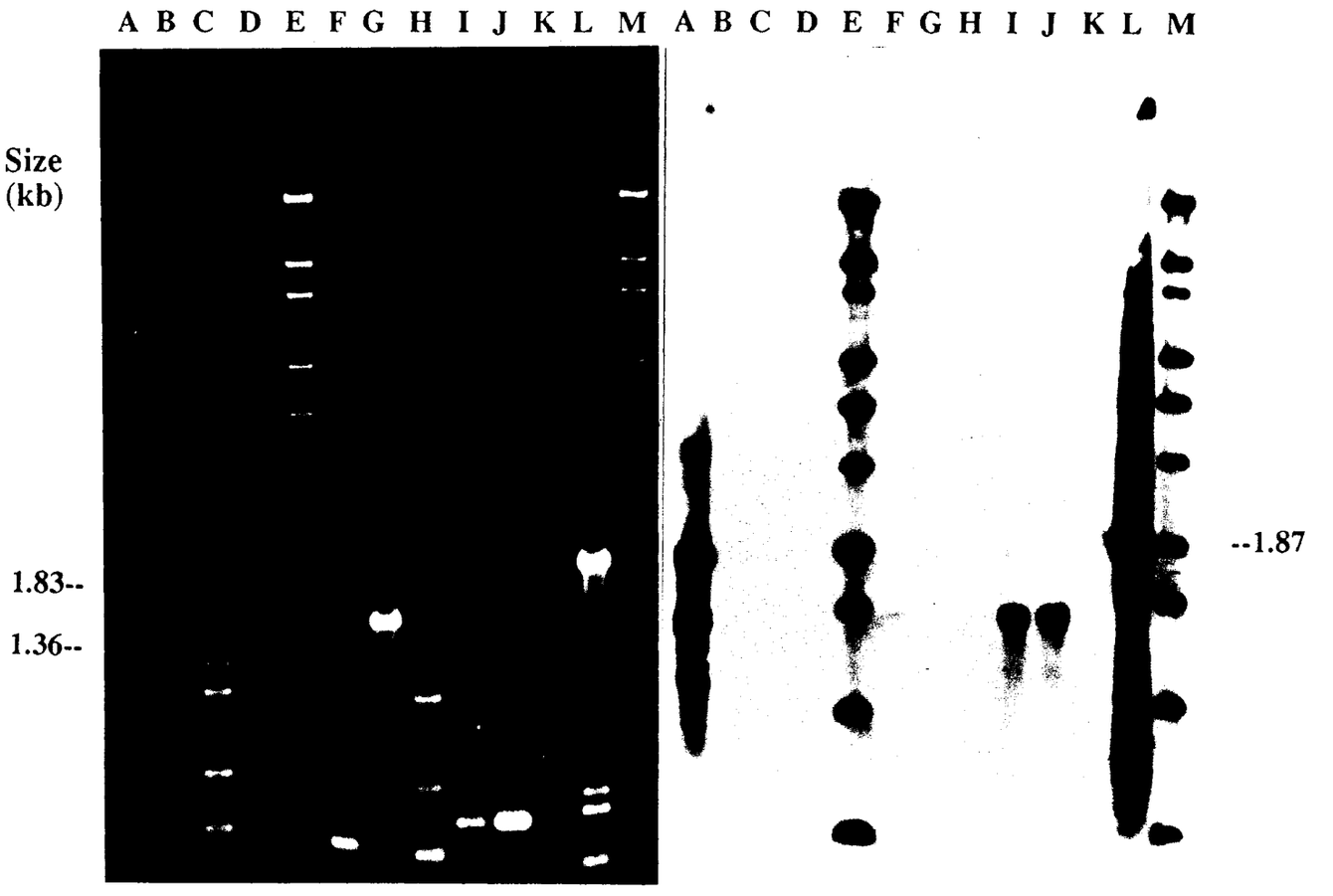

I.
II.
A B C D E F G H I J K L M
A B C D E F G H I J K L M
Size
(kb)
1.83--
1.36--
--1.87

Table 6. Summary of probes utilized and the PCR fragments to which the probes hybridized.

	Probes (Size of Hybridizing Fragment (kb))		
Strain	*E. coli* B	P58	P5
P5	1.8, 1.36		1.8, 1.36
P42			1.36
P58		1.5, 1.0	
P93	1.36		1.8, 1.36
P103	1.36		1.36
E. coli B	1.8		1.8

Figure 4. Restriction endonuclease digestions of P5 and *E. coli* strain B 1.4 Kb PCR fragments. Agarose gel electrophoresis of restriction endonuclease digestions of P5 and *E. coli* strain B (EB) 1.4 Kb PCR products. Lanes: A, AluI digest of P5 PCR DNA; B, AluI digest of EB PCR DNA; C, BstNI digest of P5 PCR DNA; D, BstNI digest of EB PCR DNA; E, BstYI digest of P5 PCR DNA; F, BstYI digest of EB PCR DNA; G, FokI digest of P5 PCR DNA; H, FokI digest of EB PCR DNA; I, HaeIII digest of P5 PCR DNA; J, HaeIII digest of EB PCR DNA; K, PstI digest of lambda DNA; L, HincII digest of P5 PCR DNA; M, HincII digest of EB PCR DNA; N, MspI digest of P5 PCR DNA; O, MspI digest of EB PCR DNA; P, Sau3AI digest of P5 PCR DNA; Q, Sau3AI digest of EB PCR DNA; R, TaqI digest of P5 PCR DNA; S, TaqI digest of EB PCR DNA;

A B C D E F G H I J K L M N O P Q R S

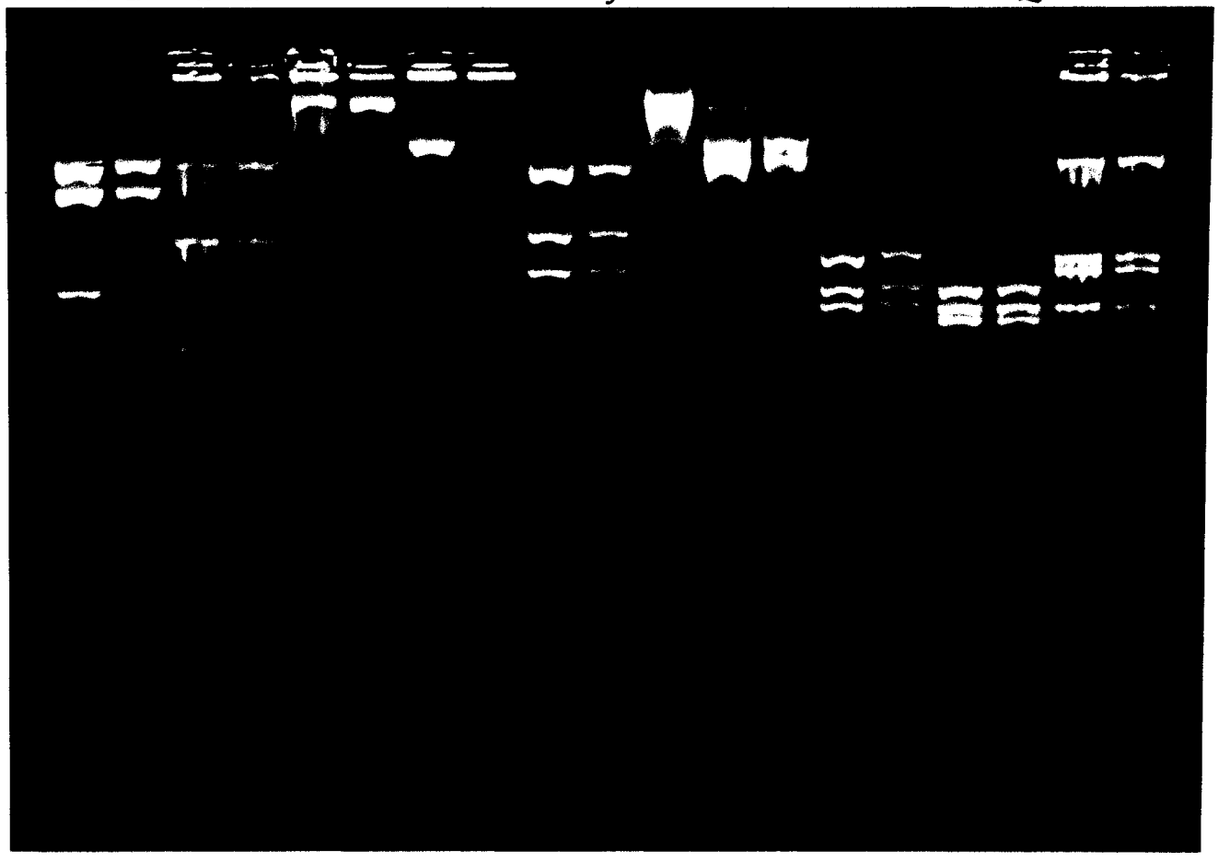
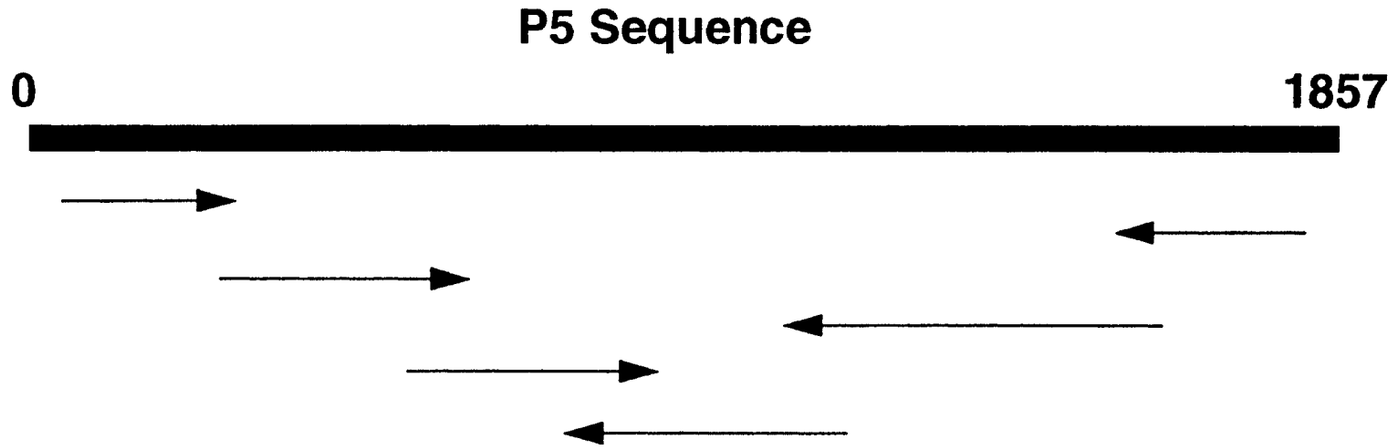


Figure 5. *Propionibacteria acidipropionici* strain P5 1.8 kb PCR fragment sequencing strategy.

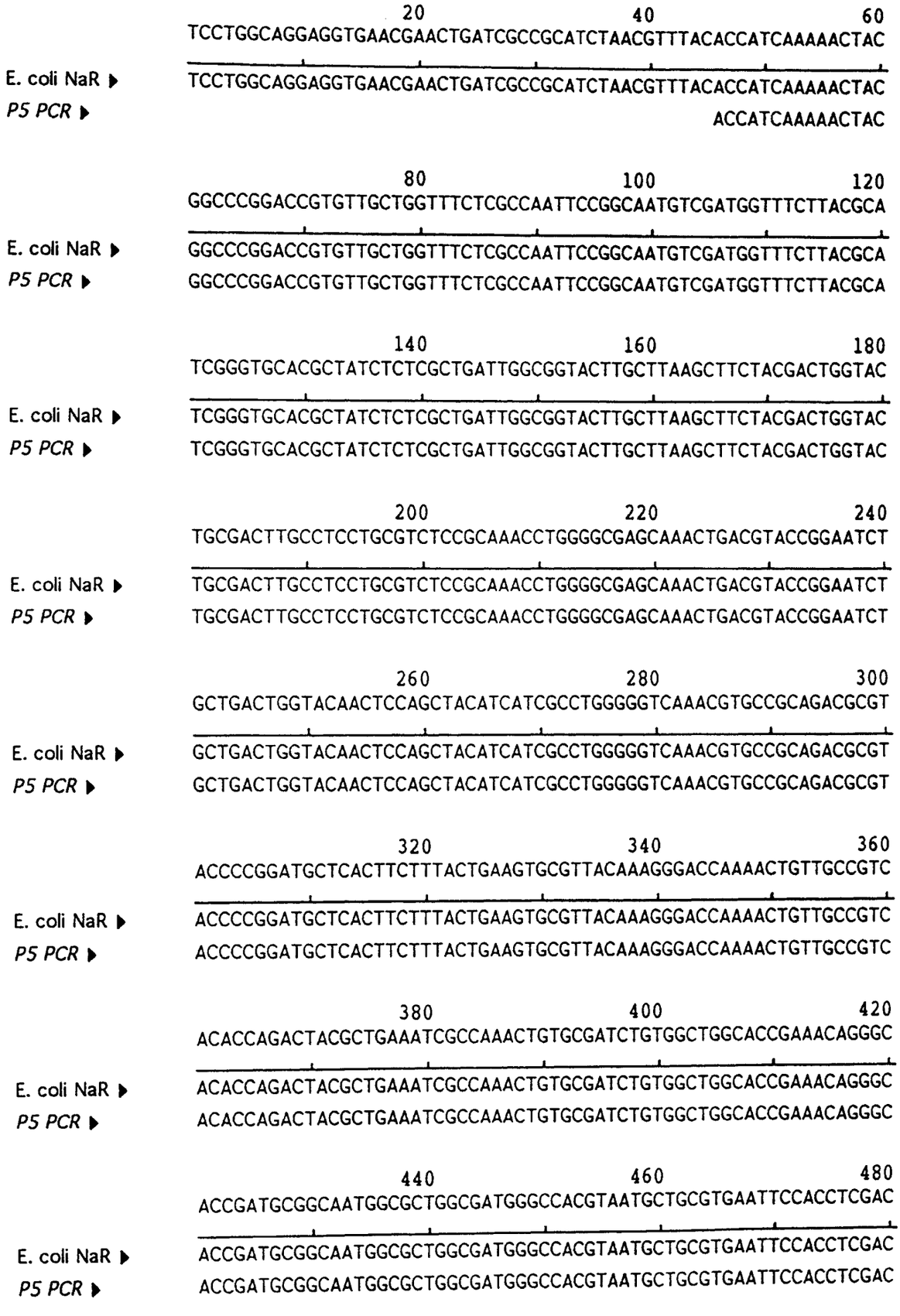

P5 Sequence
0
1857

kb PCR fragment from strain P5 containing the putative nitrate reductase fragment was identical to the *E. coli* 1.8 Kb fragment from *narG* (fig. 6). Only 7 single base differences were observed after analysis of the initial sequencing results however, on further examination of additional sequencing, these differences were not confirmed. Any remaininging differences were in sequencing regions that were not overlapped.

Confirmation of the identity of the propionibacteria strain and evidence against DNA contamination. Pulsed-field gel electrophoresis was used to confirm the identity of the propionibacteria strain used as the source of DNA for this study. Restriction enzyme digestion patterns of intact chromosomal DNA from cells retained as a frozen cell pellet were identical to restriction patterns of strain P5 (fig 7). These results indicate that the strain used as the source of template DNA for PCR reactions was in fact strain P5.

As a result of the unexpected sequencing results, additional experiments were conducted to determine if the 1.83 kb product from strain P5 was due to contamination with *E. coli* DNA. DNA extracted from two different cell pellets yielded the 1.83 and 1.36kb PCR fragments. In addition, PCR amplification of a retained DNA sample from previous work also yielded the identical 1.83 and 1.36 kb fragments.

Figure 6. *Propionibacteria acidipropionici* strain P5 1.8 kb PCR fragment sequencing data.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P5 PCR
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100
120
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P5 PCR
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180
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P5 PCR
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280
300
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E. coli NaR
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P5 PCR
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460
480
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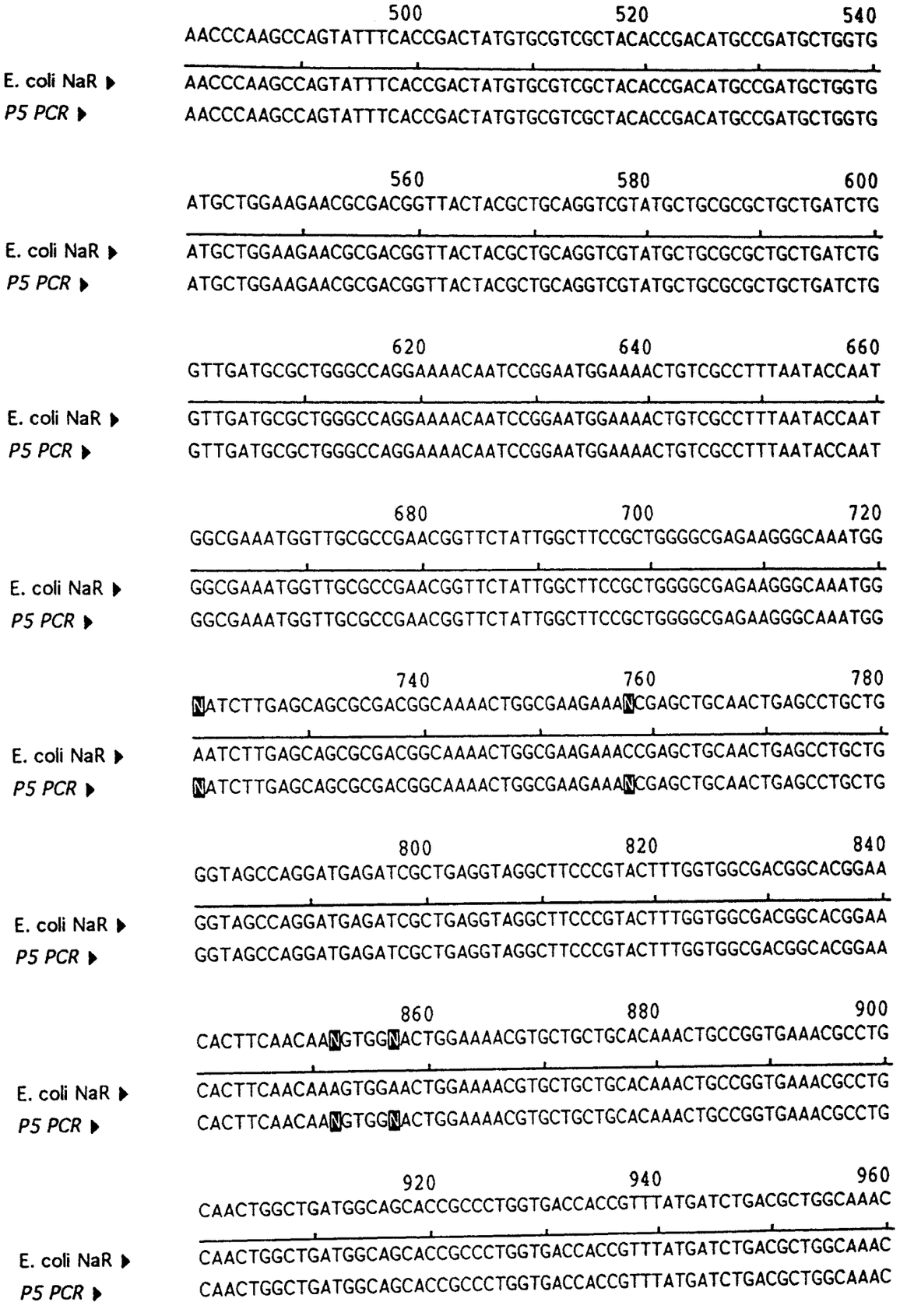
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E. coli NaR
P5 PCR
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720
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E. coli NaR
P5 PCR
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GGCGAAATGGTTGCGCCGAACGGTTCTATTGGCTTCCGCTGGGGCGAGAAGGGCAAATGG
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760
780
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E. coli NaR
P5 PCR
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840
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P5 PCR
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880
900
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E. coli NaR
P5 PCR
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920
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960
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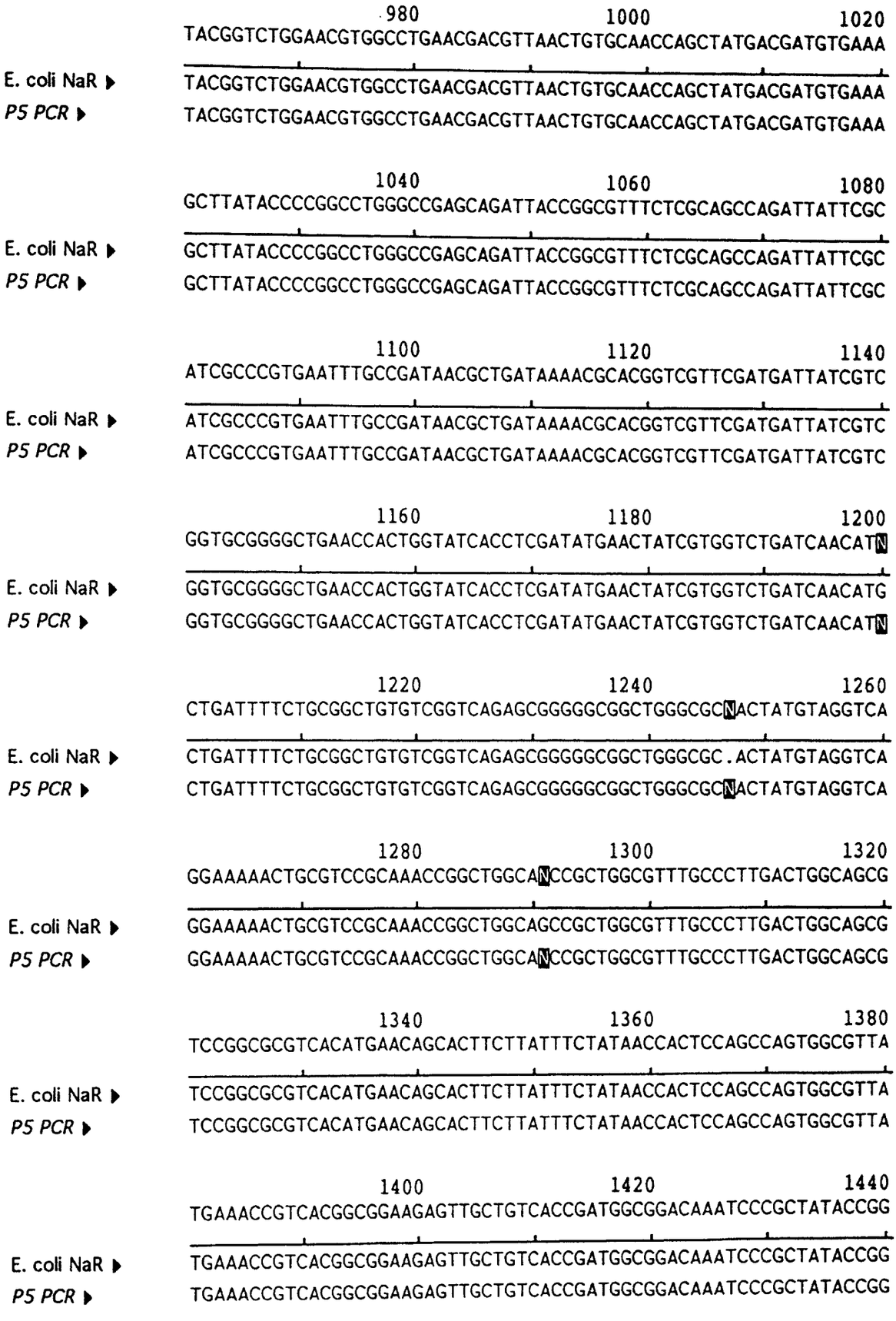
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P5 PCR
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P5 PCR
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1180
1200
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E. coli NaR
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P5 PCR
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1240
1260
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E. coli NaR
CTGATTTTCTGCGGCTGTGTCGGTCAGAGCGGGGGCGGCTGGGCGC.ACTATGTAGGTCA
P5 PCR
CTGATTTTCTGCGGCTGTGTCGGTCAGAGCGGGGGCGGCTGGGCGCNACTATGTAGGTCA
1280
1300
1320
GGAAAAACTGCGTCCGCAAACCGGCTGGCANCCGCTGGCGTTTGCCCTTGACTGGCAGCG
E. coli NaR
GGAAAAACTGCGTCCGCAAACCGGCTGGCAGCCGCTGGCGTTTGCCCTTGACTGGCAGCG
P5 PCR
GGAAAAACTGCGTCCGCAAACCGGCTGGCANCCGCTGGCGTTTGCCCTTGACTGGCAGCG
1340
1360
1380
TCCGGCGCGTCACATGAACAGCACTTCTTATTTCTATAACCACTCCAGCCAGTGGCGTTA
E. coli NaR
TCCGGCGCGTCACATGAACAGCACTTCTTATTTCTATAACCACTCCAGCCAGTGGCGTTA
P5 PCR
TCCGGCGCGTCACATGAACAGCACTTCTTATTTCTATAACCACTCCAGCCAGTGGCGTTA
1400
1420
1440
TGAAACCGTCACGGCGGAAGAGTTGCTGTCACCGATGGCGGACAAATCCCGCTATACCGG
E. coli NaR
TGAAACCGTCACGGCGGAAGAGTTGCTGTCACCGATGGCGGACAAATCCCGCTATACCGG
P5 PCR
TGAAACCGTCACGGCGGAAGAGTTGCTGTCACCGATGGCGGACAAATCCCGCTATACCGG

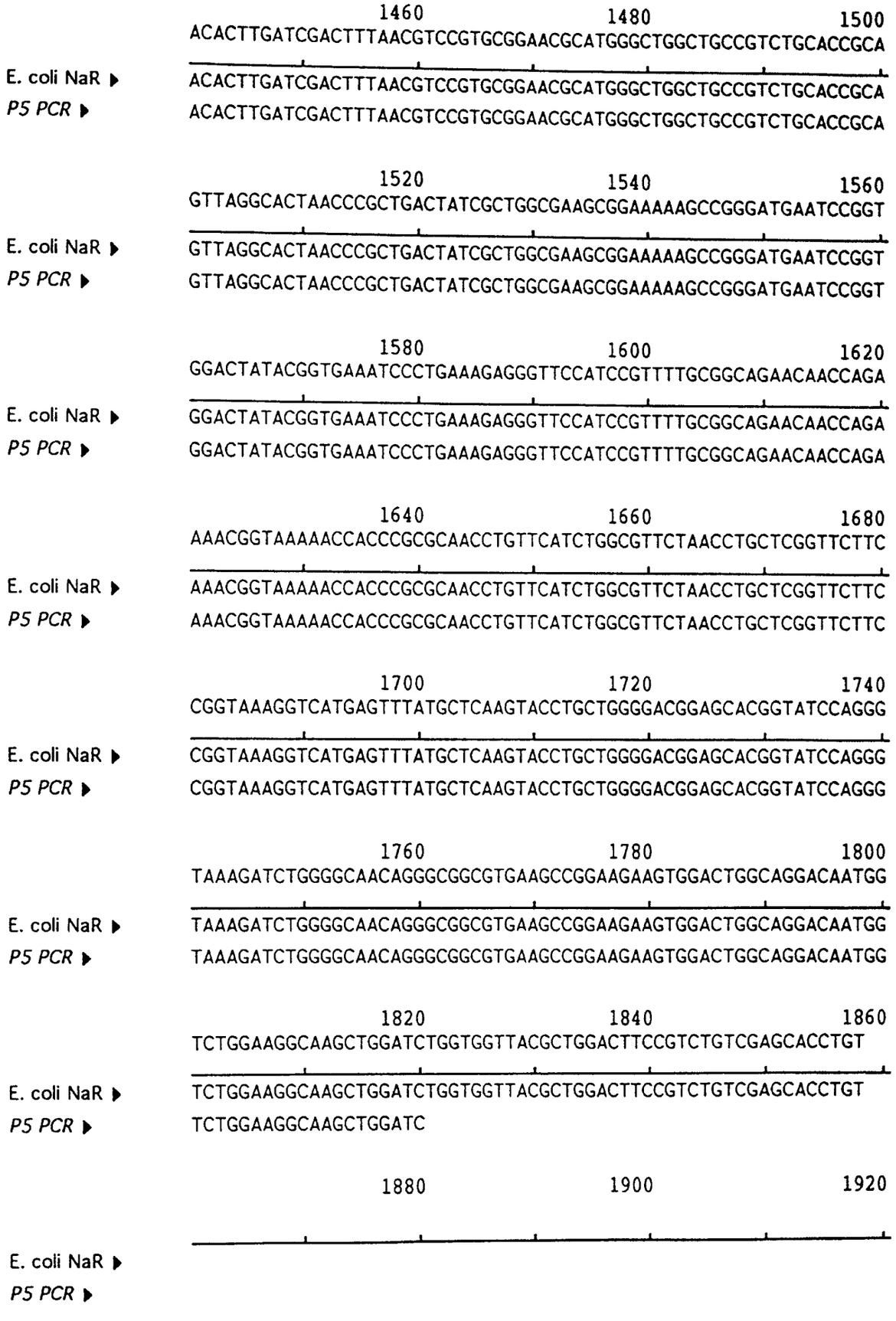
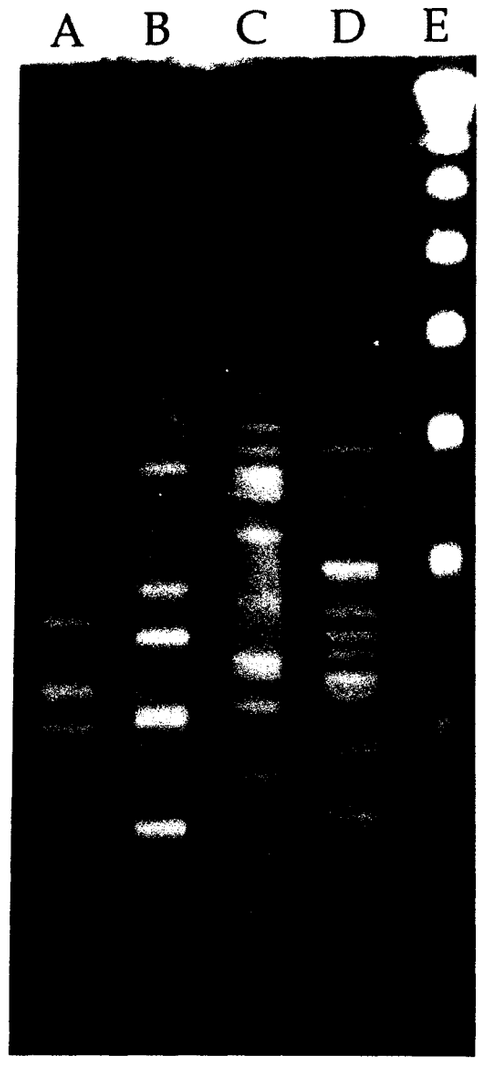

1460
1480
1500
ACACTTGATCGACTTTAACGTCCGTGCGGAACGCATGGGCTGGCTGCCGTCTGCACCGCA
E. coli NaR
ACACTTGATCGACTTTAACGTCCGTGCGGAACGCATGGGCTGGCTGCCGTCTGCACCGCA
P5 PCR
ACACTTGATCGACTTTAACGTCCGTGCGGAACGCATGGGCTGGCTGCCGTCTGCACCGCA
1520
1540
1560
GTTAGGCACTAACCCGCTGACTATCGCTGGCGAAGCGGAAAAAGCCGGGATGAATCCGGT
E. coli NaR
GTTAGGCACTAACCCGCTGACTATCGCTGGCGAAGCGGAAAAAGCCGGGATGAATCCGGT
P5 PCR
GTTAGGCACTAACCCGCTGACTATCGCTGGCGAAGCGGAAAAAGCCGGGATGAATCCGGT
1580
1600
1620
GGACTATACGGTGAAATCCCTGAAAGAGGGTTCCATCCGTTTTGCGGCAGAACAACCAGA
E. coli NaR
GGACTATACGGTGAAATCCCTGAAAGAGGGTTCCATCCGTTTTGCGGCAGAACAACCAGA
P5 PCR
GGACTATACGGTGAAATCCCTGAAAGAGGGTTCCATCCGTTTTGCGGCAGAACAACCAGA
1640
1660
1680
AAACGGTAAAAACCACCCGCGCAACCTGTTCATCTGGCGTTCTAACCTGCTCGGTTCTTC
E. coli NaR
AAACGGTAAAAACCACCCGCGCAACCTGTTCATCTGGCGTTCTAACCTGCTCGGTTCTTC
P5 PCR
AAACGGTAAAAACCACCCGCGCAACCTGTTCATCTGGCGTTCTAACCTGCTCGGTTCTTC
1700
1720
1740
CGGTAAAGGTCATGAGTTTATGCTCAAGTACCTGCTGGGGACGGAGCACGGTATCCAGGG
E. coli NaR
CGGTAAAGGTCATGAGTTTATGCTCAAGTACCTGCTGGGGACGGAGCACGGTATCCAGGG
P5 PCR
CGGTAAAGGTCATGAGTTTATGCTCAAGTACCTGCTGGGGACGGAGCACGGTATCCAGGG
1760
1780
1800
TAAAGATCTGGGGCAACAGGGCGGCGTGAAGCCGGAAGAAGTGGACTGGCAGGACAATGG
E. coli NaR
TAAAGATCTGGGGCAACAGGGCGGCGTGAAGCCGGAAGAAGTGGACTGGCAGGACAATGG
P5 PCR
TAAAGATCTGGGGCAACAGGGCGGCGTGAAGCCGGAAGAAGTGGACTGGCAGGACAATGG
1820
1840
1860
TCTGGAAGGCAAGCTGGATCTGGTGGTTACGCTGGACTTCCGTCTGTCGAGCACCTGT
E. coli NaR
TCTGGAAGGCAAGCTGGATCTGGTGGTTACGCTGGACTTCCGTCTGTCGAGCACCTGT
P5 PCR
TCTGGAAGGCAAGCTGGATC
1880
1900
1920
E. coli NaR
P5 PCR

Figure 7. Restriction endonuclease digestions of *Propionibacterium acidipropionici* strain *P5* chromosomal DNA. TAFE II separation of P5 chromosomal DNA fragments. Lanes: A, AsnI digest of P5 chromosomal DNA; B, HpaI digest of P5 chromosomal DNA; C, SnaB I digest of P5 chromosomal DNA; D, XbaI digest of P5 chromosomal DNA; E, Lambda ladder molecular size standard.


A
B
C
D
E

Discussion

We describe here the use of the polymerase chain reaction, southern hybridizations, DNA sequencing, and restriction endonuclease digestions to characterize the nitrate reductase gene of *Propionibacterium*. PCR amplification of *E. coli* DNA using primers based on the *E. coli* nitrate reductase sequence produced the predicted 1.87 kb fragment of *narG*. PCR amplification of DNA from 10 propionibacteria strains yielded only a single visible fragment in agarose gels from one strain (P58). However, sequential amplification of the first PCR reaction did produce detectable fragments from all propionibacteria strains. DNA impurity or inefficient primer annealing due to lack of complementary sequences at the primer binding sites may explain the poor yields from the initial propionibacteria amplification of the 1.83 kb fragment, thus producing fragments that were not visible in agarose gels. These results lead us to believe that inefficiencies in primer annealing process were the primary problem.

Sequential PCR amplification of propionibacteria strain P5 produced a visible fragment that was nearly identical in size to the *E. coli narG* PCR product. No other propionibacteria strain produced a visible fragment of comparable size. PCR amplification of the remaining eight strains yielded a variety of fragments in number and size. No readily identifiable fragment pattern was produced among strains of the same species. These results may indicate some degree of heterologeneity in the nitrate reductase of propionibacteria. Alternatively, the variety of fragments produced by PCR maybe due to non-specific amplification of other unknown DNA regions.

Southern hybridization revealed that similar sequences were present in the *E. coli* 1.87 kb *narG* fragment and the 1.83 kb fragment from strain P5 as well as the 1.36 kb fragment from strains P5, P93 and P103. However, none of the remaining nitrate positive or denitrifying propionibacteria strains produced DNA fragments that hybridized at high stringency to this *narG* probe. These results seem to indicate an evolutionary divergence of the nitrate reductase in propionibacteria.

In a reciprocal hybridization experiment, the 1.83 kb P5 probe strongly hybridized to 1.87 kb *E. coli* fragment and the remaining fragments detected by the *E. coli* probe. In addition, the P5 probe weakly hybridized to a 1.36 kb fragment from P42 and a 1.8 kb fragment from P93. Whether the additional fragments detected using the 1.83 kb P5 probe are significant to distinguish this probe from the *E. coli* probe is probably not true given that the signal was extremely faint and was only detected by extended exposure times. This assumption was later confirmed based on the DNA sequencing results.

The hybridization of the *narG* probe to the 1.36 fragment in P5 and the two other propionibacteria strains was unexpected. This fragment may be a truncated form of the 1.8 kb product or an additional conserved sequence encoding another molybdoenzyme or additional nitrate reductase as identified in *E. coli* (Blasco et al., 1989;1990).

The results of the hybridization of the P58 probe are puzzling given the fact that this was the only strain to produce a visible fragment after an initial PCR amplification using the primers from the *E. coli* nitrate reductase. Based on the success of the PCR amplification, we initially had thought that this strain contained a nitrate reductase related to *E. coli*. However, results of hybridization experiments indicated that this fragment contains a unique DNA sequence. The identity of this sequence is presently unknown.

Sequencing of the propionibacteria strain P5 1.8 kb PCR product demonstrated a 100% homology between the product and the published *Escherichia coli* K12 sequence data. Although we assumed based on the PCR results that the internal regions were conserved in the P5 nitrate reductase, we assumed that the internal regions would have

only a limited degree of homology to *E. coli* at the DNA level. This would be suggested given slight differences in GC content between the two organisms. However the limited amount of propionibacteria sequence information does not allow assumptions to be made about the preferred codon usage of these organisms. To date only two studies have been published in which sequence information has been derived from propionibacteria genes (Murakami et al., 1993; Ladror et al., 1991). The selection pressures for conserved sequences are at the level of protein not DNA, thus one would assume that the DNA sequence would drift apart even if the amino acid sequence remained the same. This conservation phenomenon is not, however, unprecedented. The shiga toxin of *Shigella dysenteriae* and the shiga-like toxin of *Escherichia coli* have 1.4 kb nucleotide sequences that are more than 99% homologous (Strockbine et al., 1988). Three nucleotide differences were detected in three separate codons, with only one amino acid being affected. Perhaps further sequencing of the entire P5 nitrate reductase gene would detect differences lying outside of the highly conserved molybdenum binding site encoding regions. The 1.8 kb region that was sequenced is less than 1/3 of the reported 7 kb size of the *E. coli* nitrate reductase operon.

The unexpected 100% sequence similarity of the P5 1.8 kb PCR fragment to that of the *E. coli narG* gene lead us to consider the possibility of DNA contamination. The fact that three separate sources of P5 chromosomal DNA produce the same 1.8 and 1.35 kb fragments and PCR controls did not produce visible fragments, greatly diminishes the possibility of *E. coli* chromosomal DNA contamination. Regardless of strong support of evidence that the 1.8 kb sequence is truly amplified from P5 chromosomal DNA one cannot rule out the possiblity of contamination.

References

Antipov, V., and V. Subbotin. 1980. Use of probiotics-efficacy and prospects. Ref. Dairy Sci. Abstr. 1982, 44(11):8567.

Biede, S., and E. Hammond. 1979. Swiss cheese flavor. II. Organoleptic analysis. J. Dairy Sci. **62**:238-248.

Cerna, J. 1984. Elvit, fermented milk beverage. Prum.-Potravin. **35**:192-196.

Dawson, T., L. Miranda, M. Yokoyama and S. Rust. 1991. Inoculation of reconstituted high moisture corn with propionic acid producing bacteria with or without the additon of lactic acid bacteria. 21st Biennial Conference on Rumen Function. Chicago, IL. November, 1991. (Abst. 62)

Flores-Galarza, R., B. Glatz, J. Bern, and L. van Fossen. 1985. Preservation of high-moisture corn by microbial fermentation. J. Food Prot. **48**:407-411.

Grinstead, D., and S. Barefoot. 1992. Jenseniin G, a heat-stable bacterocin produced by *Propionibacterium jensenii* P126. Appl. Environ. Microbiol. **58**:215-220.

Hofherr, L., and B. Glatz. 1983. Mutagenesis of strains of propionibacteria to produce cold sensitive mutants. J. Dairy Sci. **66**:2482-2487.

Kauc. L., M. Mitchell, and S. Goodgal. 1989. Size and physical map of the chromosome of *Haemophilus influenzae*. J. Bacteriol. **171**:2474-2479.

Kemp, A., J. Geurink, Haalstra, and A. Malestein. 1977. Nitrate poisoning in cattle. 2. Changes in nitrite in rumen fluid and methemoglobin formation after high nitrate intake. Neth. J. agric. Sci. **25**:51-62.

Kemp, A., J. Geurink, A. Malestein, and A. van't Klooster. 1978. Grassland production and nitrate poisoning in cattle. Proc. 7th gen. Meet. Eur. Grassld. Fedn. (Gent).

Kornyeva, V. 1981. Establishment of propionic acid bacteria in the intestine of infants given propioni-acidophilus milk. Ref. Dairy Sci. Abstr. 1982, **44**:420.

Ladror, U., L. Gollapudi, R. Tripathi, S. Latshaw, and P. Kemp. 1991. Cloning, sequencing, and expression of pyrophosphate-dependent phosphofructokinase from *Propionibacterium freudenreichii*. J. Biol. Chem. **266**:16550-16555.

Langsrud, T., and G. Reinbold. 1973. Flavor development and mcirobiology of Swiss cheese-a review. II. Starters, manufacturing processes and procedures. J. Milk Food Technol. **36**:531-542.

Langsrud, T., and G. Reinbold. 1973. Flavor development and microbiology of Swiss cheese a review. IV. Defects. J. Milk Food Technol. **37**:24-41.

Lyon, W., and B. Glatz. 1991. Partial purification and characterization of a bacteriocin produced by *Propionibacterium theonii*. Appl. Environ. Microbiol. **57**:701-706.

Maniatis, T., E. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Mantere-Alhonen, S. 1982. Die Propionibakterien der Molkereiindustrie als Darmkanalmikroben. Meijeritieteellinen Aikakauskirja. **XL** (1) 95 S.

Mantere-Alhonen, S. 1983. On the survival of a *Propionibacterium freudenreichii*-culture during 'in vitro' gastric digestion. Meijeritieteellinen Aikakauskirja. **XLI**:19-23.

Mantere-Alhonen, S., and E. Makinen. 1987. A new type of sour milk with *Propionibacteria*. Meijeritieteellinen Aikakauskirja. XLV:49-61.

Mantere-Alhonen, S., and H. Mllymaki. 1985. A substrate for the production of propionibacteriamass. Kemia-Kemi short communications, n:o 11.

Meyers, J., D. Sanchez, L. Elwell, and S. Falkow. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. **127**:1529-1537.

Murakami, K., Y. Hashimoto, and Y. Murooka. 1993. Cloning characterization of the gene encoding glutamate 1-semialdehyde 2,1-aminomutase, which is involved in δ-Aminolevulinic acid synthesis in *Propionibacterium freudenreichii.* Appl. Environ. Microbiol. **59**:347-350.

Nabukhotnyi, T., S. Cherevko, G. Sanigullina, and A. Grushko. 1985. Use of adapted propioni-acidophilic "Malyutka" and "Malyush" formulas in complex treatment of acute gastrointestinal diseases of infants. Ref. Dairy Sci. Abstr. 1985, **47**:2122.

Perlman, D. 1978. p.303-326. In: Rose, A. H. (ed.) Economic microbiology, vol. 2., Primary products of metabolism. Academic Press Inc., London.

Playne, M. 1985. p.731-759. Propionic and butyric acids. In: Moo-Young, M. (ed.) Comprehensive biotechnology. Pergamon Press, Oxford.

Raeker, M., C., Bern, L. Johnson and B. Glatz. 1992. Preservation of high-moisture maize by various propionate treatments. Cereal Chem. **69**:66-69.

Rehberger, T. 1984. Thesis. Iowa State University

Rehberger, T. 1993. Genome analysis of *Propionibacterium freudenreichii* by pulsed-field electrophoresis. Curr. Microbiol. **27**:21-25.

Rehberger, T., C. Hibberd, and J. Swartzlander. 1993. p.E1-E15. Identification and application of a propionibacteria strain for nitrate and nitrite reduction in the rumen. In: Management of high nitrate forages for beef and dairy cattle, G. Highfill (Ed.) Okla. Coop. Ext. Serv., Enid.

Sanger, F., S. Nicklen, A. Coulson. 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA. **74**:1342-1346.

Sanigullina, F. I., and W. Pawliuk. 1984. Eine experimentelle beurteilung der biologishchen wertigkeit der Propionsaure Acidophilus-Nahrmittel "Malyutka" und "Malyush". Ref. Mi. 1985, 585.

Sidorchuk, I., and V. Bondarenko. 1984. Selection of a biologically active mutant of *Propionibacterium shermanii* and the possibility of its use in complex therapy of enteral dysbacteriosis. J. Hyg. Epidemiol. Microbiol. Immunol. **28**:331-333.

Sidorchuk, I., V. Bondareko, B. Patraty, S. Cherevko, and T. Nabukhotny. 1984. Efficiency of biopreparation from *Propionibacterium shermanii* in prophylaxis of dysbacteriosis in salmonellosis and shigellosis treatment . Mikrobiol. ZH. **46**:43-46.

Somkuti, G., and T. Johnson. 1990. Cholesterol uptake by *Propionibacterium freudenreichii*. Curr. Microbiol. **20**:305-309.

Southern, E. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503-517.

Stockbine, N., M. Jackson, L. Sung, R. Holmes, A. O'Brien. 1988. Cloning and sequencing of the genes for shiga toxin from *Shigella dysenteriae* type 1. J. Bacteriol. **170**:1116-1122.

Tomes, N. 1991. Bacterial treatment to preserve silage. US Patent 4,981,705.

Tuikov, R. M. Khristova, K. Rizanvov, and I. Vladimirov. 1980. Early weaning of calves using a milk additive and a preparation of freeze-dried bacteria. Dairy Sci. Abstr. 1982, 45 (4):1689.

Vladimirov, I., K. Rizanvov, A. Vasilev, and P. Kenov. 1977. Tests on replacement of antibiotics in sucking calf feeding by a freeze-dried preparation of bacteria. Dairy Sci. Abstr. 1978. **40** (4):2118.

Woolford, M. 1975. The significance of *Propionibacterium* species and *Micrococcus lactilyticus* to the ensiling process. J. Appl. Bacteriol. **39**:301-306.

CHAPTER IV

SUMMARY AND CONCLUSIONS

The genes encoding the nitrate reducing system of ten *Propionibacterium* strains were examined in this investigation. Using conserved sequences within a number of the *E. coli* molybdoenzymes including the nitrate reductase, PCR primers were designed. PCR amplification conditions and mixture concentrations were optimized, allowing the amplification of various products from the ten *Propionibacterium* strains. Southern hybridizations indicate that products from three of the propionibacteria strains (P5, P93, P103) have DNA sequence similarity to a product that was amplified from the *E. coli* nitrate reductase *nar*G gene. Of these strains, two of the strains (P5, P93) had products amplified that had DNA sequence similarity to the *E. coli narG* product but were also similar in size to the 1.8 kb *E. coli* product. The 1.8 kb product of propionibacteria strain P5 was analyzed using restriction endonuclease digestions and DNA sequencing. Digestion patterns between the P5 and *E. coli* PCR products were found to be identical. DNA sequence comparisons of the propionibacteria strain P5 to *E. coli narG* published data revealed that these sequences were identical. Pulse-field gel electrophoresis, repeated PCR amplifications and Southern hybridizations of different chromosomal DNA isolations confirmed that our results were not due to contamination.

Results of PCR amplification and Southern hybridization indicate that there are at least two types of genetically encoded nitrate reductase systems within the ten nitrate reducing *Propionibacterium* strains examined. One systems is amplified using primers designed from the nitrate reductase of *E. coli* and additionally hybridizes to a PCR

product amplified from the *narG* gene of the *E. coli* nitrate reductase. Three of the propionibacteria strains (P5, P93, P103) are grouped within the first nitrate reducing system. The remainder of the propionibacteria strains examined appear to have nitrate reducing genes that do not have DNA sequence similarities to that of the *E. coli* nitrate reductase. Restriction endonuclease digestion patterns and sequence data from strain P5 indicate the nitrate reducing gene similarities between that of a propionibacteria strain and *E. coli*. The DNA sequence similarities between these two strains may indicate that a recent genetic exchange has occurred between these two organisms.

Further examination of the propionibacteria denitrification system will provide explanations about the possibility of the relatedness of the *E. coli* and P5 nitrate reductase structure and function. This may provide the possibility of reducing the toxic effects of nitrate-nitrite in cattle through classical strain manipulations and genetic engineering. In addition, further examination may provide information about a gram-positive membrane bound enzyme that has been studied extensively in gram-negative bacteria.

Comprehensive Bibliography

Abbit, B. 1982. A case of nitrate induced abortion in cattle. Southwestern Vet. **35**:12.

Allison, C., and G. MacFarlane. 1989. Dissimilatory nitrate reduction by *Propionibacterium acnes*. Appl. Environ. Microbiol. **55**:2899-2903.

Anderson, T. 1985. Mycostatic whey and process of manufacture. US Patent 4,497,833.

Antipov, V. A., and V. M. Subbotin. 1980. Use of probiotics-efficacy and prospects. Ref. Dairy Sci. Abstr. 1982, 44(11):8567.

Arnst, H. Jr., D. MacDonald, and D. Cove. 1970. Molybdate metabolism in *Aspergillus nidulans*. I. Mutations affecting nitrate reductase and/or xanthine dehydrogenase. Mol. Gen. Genet. **108**:129-145.

Biede, S., and E. Hammond. 1979. Swiss cheese flavor. II. Organoleptic analysis. J. Dairy Sci. **62**:238-248.

Bilous, P., S. Cole, W. Anderson, and J. Weiner. 1988. Nucleotide sequence of the *dms*ABC operon encoding the anaerobic dimethylsulfoxide reduction of *Escherichia coli*. Mol. Microbiol. **2**:785-795.

Blasco, F., C. Iobbi, G. Giordano, M. Chippaux, and V. Bonnefoy. 1989. Nitrate reductase of *Escherichia coli*: Completion of the nucleotide sequence of the *nar* operon and reasessment of the role of the alpha and beta subunits in iron binding and electron transfer. Mol. Gen. Genet. **218**:249-256.

Blasco, F., C. Iobbi, J. Ratouchniak, V. Bonnefoy, and G. Chippaux. 1990. Nitrate reductases of *Escherichia coli*: Sequence of the second nitrate reductase and comparison with that encoded by the *nar*GHJI operon. Mol. Gen. Genet. **222**:104-111.

Bonnefoy, V., J. Burini, G. Giordano, M. Pascal, and M. Chippaux. 1987. Presence in the "silent" terminus region of the *Escherichia coli* K12 chromosome of cryptic gene(s) encoding a new nitrate reductase. Mol. Microbiol. **1**:143-150.

Bonnefoy-Orth, V., M. Lepelletier, M. Pascal, and Chippaux, M. 1981. Nitrate reductase and cytochrome $b_{\text{nitrate reductase}}$ structural genes as parts of the nitrate reductase operon. Mol. Gen. Genet. **181**:535-540.

Broekhoven, L., and R. Stephany. 1978. Environmental aspects of N-nitroso compounds. IARC Scientific Publications: 19.

Brumm, P., and R. Datta. 1989. Production of organic acids by an improved fermentation process. US Patent 4,814,273.

Burrows, G., G. Horn, R. McNew, L. Croy, R. Keeton, and J. Kyle. 1987. The prophylactic effect of corn supplementation on experimental nitrate intoxication in cattle. J. Anim. Sci. **64**:1682-1689.

Cerna, J. 1984. Elvit, fermented milk beverage. Prum.-Potravin. **35**:192-196.

Chaudhry, G., and C. MacGregor. 1983a. *Escherichia coli* nitrate reductase subunit A: its role as the catalytic site and evidence for its modification. J. Bacteriol. **154**:387-394.

Chaudhry, G., and C. MacGregor. 1983b. Cytochrome *b* from *Escherichia coli* nitrate reductase. Its properties and association with the enzyme complex. J. Biol. Chem. **258**:5819-5827.

Chiang, R., C. Cavicchioli, and R. P. Gunsalus. 1992. Identification and characterization of *narQ*, a second nitrate sensor for nitrate-dependent gene regulation in *Escherichia coli*. Mol. Microbiol. **6**:1913-1923.

Chippaux, M., V. Bonnefoy-Orth, J. Ratouchniak, and M. Pascal. 1982. Operon fusion in the nitrate reductase operon and study of the control gene *nir*R in *Escherichia coli*. Mol. Gen. Genet. **182**:477-479.

Chippaux, M., and F. Pichinoty. 1970. Les nitrate-reductases bacteriennes. V. Induction de la biosynthese de l,enzyme A par l'azoture. Archiv fur Mikrobiologie. **71**:361-366.

Clegg, R. 1976. Purification and some properties of nitrate reductase (E.C. 1-7-99-4) from *Escherichia coli* K12. Biochem. J. **153**:533-541.

Crueger, W., and A. Crueger. 1982. Biotechnology: a textbook of industrial microbiology. Science Tech. Inc. Madison, WI.

Cummins, C., and J. Johnson. 1986. Section 15. Propionibacterium, pp. 1346-1353. *In* Peter H. A. Sneath, (ed.); Bergey's Manual of Systematic Bacteriology, Vol. 2. The Williams and Wilkins Co., Baltimore, MD.

Dawson, T., L. Miranda, M. Yokoyama and S. Rust. 1991. Inoculation of reconstituted high moisture corn with propionic acid producing bacteria with or without the additon of lactic acid bacteria. 21st Biennial Conference on Rumen Function. Chicago, IL. November, 1991. (Abst. 62)

DeMoss, J. A. 1977. Limited proteolysis of nitrate reductase purified from membranes from *Escherichia coli*. J. Biol. Chem. **252**:1696-1701.

DeMoss, J., T. Fan, and R. Scott. 1981. Characterization of subunit structural alterations which occur during purification of nitrate reductase from *Escherichia coli*. Arch. Biochem. Biophys. **206**:54-64.

DeMoss, J., and P. Hsu. 1991. NarK enhances nitrate uptake and nitrite excretion in *Escherichia coli*. J. Bacteriol. **173**:3303-3310.

Der Vartanian, D., and P. Forget. 1975. The bacterial nitrate reductase EPR studies on the enzyme A of *Escherichia coli* K12. Biochim. Biophys. Acta. **379**:74-80.

DeVries W., M. Aleem, A. Hemrika-Wagner, and A. Stouthamer. (1977). The functioning of cytochrome b in the electron transport to fumarate in *Propionibacterium freudenreichii* and *P. pentosaceum*. Arch. Microbiol. **112**:271-276.

DeVries, W., W. van Wijck-Kapteign and A. H. Stouthamer. 1972. Influence of oxygen on growth, cytochrome synthesis and fermentation pattern in propionic acid bacteria. J. Gen. Microbiol. **71**:515-524.

Dong, X., J. Li, and J. DeMoss. (1992). Upstream elements required for NarL-mediated activation of transcription from the narGHJI promoter of *Escherichia coli*. J. Biol. Chem. **263**:14122-14128.

Downey, R., and Kiszkiss, D. 1969. Oxygen and nitrate induced modification of the electron transfer system of *Bacillus stearothermophilus*. Microbios. **2**:145-153.

Duwat, P., S. Ehrlich, and A. Gruss. 1992. Use of degenerate primers for polymerase chain reaction cloning and sequencing of the *Lactococcus lactis* subsp. *lactis recA* Gene. Appl. Environ. Microbiol. **58**:2674-2678.

Edwards, E., S. Rondeau, J. DeMoss. 1983. *chlC*(*nar*) operon of *Escherichia coli* includes genes for alpha and beta subunits of the nitrate reductase. J. Bacteriol. **153**:1513-1520.

Enoch, H., and R. Lester. 1972. Effects of molybdate, tungstate, and selenium compounds on formate dehydrogenase and other enzyme systems in *Escherichia coli*. J. Bacteriol. **110**:1032-1040.

Enoch, H., and R. Lester. 1974. The role of a novel cytochrome b containing nitrate reductase and quinone in the in vivo reconstruction of formate-nitrate reductase activity of *Escherichia coli*. Biochem. Biophys. Res. Commun. **61**:1234-1241.

Enoch, H., and R. Lester. 1975. The purification and properties of formate dehydrogenase and nitrate reductase from *Escherichia coli*. J. Biol. Chem. **250**:6653-6705.

Flores-Galarza, R., B. Glatz, J. Bern, and L. van Fossen. 1985. Preservation of high-moisture corn by microbial fermentation. J. Food Prot. **48**:407-411.

Forget, P. 1971. Les nitrate-reductases bacteriennes. Solublisation, purification et proprietes de l'enzyme A de *Micrococcus denitrificans*. Eur. J. Biochem. **18**:442-450.

Forget, P. and D. Der vartanian. 1972. The bacterial nitrate reductases: EPR studies on nitrate reductase A from *Micrococcus denitrificans*. Biochim. Biophys. Acta. **256**:600-606.

Forget, P. 1974. The bacterial nitrate reductases. Solubilization, purification and properties of the enzyme A of *Escherichia coli* K12. Eur. J. Biochem. **42**:325-332.

Garland, P., R. Clegg, D. Boxer, J. Downie, and B. Haddock. 1975. In E. Quagliariello et al. (ed.) Electron transport and oxidative phosphorylation. p. 351-358. North Holland, Amsterdam.

Gent-Ruijters, M. van, van W. de Vries and A. Stouthamer. 1975. Influence of nitrate on fermentation pattern, molar growth yields and synthesis of cytochrome *b* in *Propionibacterium pentosaceum*. J. Gen. Microbiol. **88**:36-48.

Geurink, J., A. Malestein, A. Kemp, and A. van't Klooster. 1979. Nitrate poisoning in cattle. 3. The relationship between nitrate intake with hay or fresh roughage and the speed of intake on the formation of methemoglobin. Neth. J. argic. Sci. **27**:268-276.

Gilmour, C., R. Bhatt, and J. Mayeux. 1964. Comparative role of nitrate and molecular oxygen in the dissimilation of glucose. Nature, London. **203**:55-58.

Glaser, J., and J. DeMoss. 1971. Phenotypic restoration by molybdate of nitrate reductase activity in chlD mutants of *Escherichia coli*. J. Bacteriol. **108**:854-860.

Graham, A., and D. Boxer. 1980a. Implication of alpha-subunit of *Escherichia coli* nitrate reductase in catatlytic activity. Biochem. Soc. Trans. **8**:329-330.

Graham, A., and D. Boxer. 1980b. The membrane location of the beta subunit of nitrate reductase from *Escherichia coli*. Biochem. Soc. Trans. **8**:331.

Grinstead, D., and S. Barefoot. 1992. Jenseniin G, a heat-stable bacterocin produced by *Propionibacterium jensenii* P126. Appl. Environ. Microbiol. **58**:215-220.

Groot, G. de and A. Stouthamer. 1969. Regulation of reductase formation in *Proteus mirabilis*. I. Formation of reductases and enzymes of the formic hydrogenlyase complex in the wild type and in chlorate-resistant mutants. Archiv fur Mikrobiologie. **66**:220-233.

Groot, G. de, and A. Stouthamer. 1970a.. Regulation of reductase formation in *Proteus mirabilis*. II. Influence of growth with azide and of haem deficiency on nitrate reductase formation. Biochim. Biophys. Acta. **208**:414-427.

Groot, G. de, and A. Stouthamer. 1970b. Regulation of reductase formation in *Proteus mirabilis*. III. Influence of oxygen, nitrate and azide on thiosulfate reductase and tetrathionate reductase formation. Archiv fur Mikrobiologie. **74**:326-329.

Hadjipetrou, L., and A. Stouthamer. 1965. Energy production during nitrate respiration by *Aerobacter aerogenes*. J. Gen. Microbiol. **38**:29-34.

Hartingsveldt, J. van, and A. Stouthamer. 1973. Mapping and characterization of mutants of *Pseudomonas aeruginosa* affected in nitrate respiration in aerobic or anaerobic growth. J. Gen. Microbiol. **74**:97-106.

Hettinga, D., and G. Reinbold. 1975. Split defect of Swiss cheese. II. Effect of low temperatures on the metabolic activity of *Propionibacterium*. J. Milk Food Technol. **38**:31-35.

Hettinga, D., G. Reinbold, and E. Vedamuthu. 1974. Split defect of Swiss cheese. I. Effect of strain of *Propionibacterium* and wrapping material. J. Milk Food Technol. **37**:322-328.

Hibbs, C. M., E. Stencel, and R. Hill. 1978. Nitrate toxicosis in cattle. Vet. and Human Toxicology. **20**:1-2.

Hofherr, L., and B. Glatz. 1983. Mutagenesis of strains of propionibacteria to produce cold sensitive mutants. J. Dairy Sci. **66**:2482-2487.

Hudson, D., and J. Rawls. 1992. Nitrate toxicity in commercial beef herd. Nebraska Cattleman. **48**:58.

Iida, K., and S. Taniguchi. 1959. Studies on nitrate reductase system of *Escherichia coli*. Particulate electron transport system to nitrate and its solubilization. J. Biochem. **46**:1041-1055.

Iobbi, C., C. Santini, V. Bonnefoy, and G. Giordano. 1987. Biochemical and immunological evidence for a second nitrate reductase in *Escherichia coli* K12. Eur. J. Biochem. **168**:451-459.

Iobbi-Nivol, C., C. Santini, F. Blasco, and G. Giordano. 1990. Purification and characterization of the second nitrate reductase of *Escherichia coli* K-12. J. Biochem. **188**:679-687.

Ishizuka, M., T. Toraya, and S. Fukui. 1984. Purification, properties and limited proteolysis of nitrate reductase from *Pseudomonas denitrificans*. Biochem. Biophys. Acta. **786**:133-143.

Itagaki, E., T. Fujita, and R. Sato. 1962. Solubilization and properties of formate dehydrogenase and cytochrome b_1 from *Escherichia coli*. J. Biochem. **52**:131-141.

Iuchi, S., and E. Lin. 1987. The *narL* gene product activates the nitrate reductase operon and represses the fumarate reductase and trimethylamineN-oxide reductase operons in *Escherichia coli*. Proc. Natl. Acad. Sci. USA. **84**:3901-3905.

Kalman, L., and R. Gunsalus. 1988. The *frdR* gene of *Escherichia coli* globally regulates several operons involved in anaerobic growth in response to nitrate. J. Bacteriol. **170**:623-629.

Kalman, L., and R. Gunsalus. 1989. Identification of a second gene involved in global regulation of fumarate reductase and other nitrate-controlled genes for anaerobic respiration in *Escherichia coli*. J. Bacteriol. **171**:3810-3816.

Kalman, L., and R. Gunsalus. 1990. Nitrate and molybdenum-independent signal transduction mutations in *narX* that alter regulation of anaerobic respiratory genes in *Escherichia coli*. J. Bacteriol. **172**:7049-7056.

Kaneko, M., and M. Ishimoto. (1978) A study on nitrate reductase from *Propionibacterium acidi-propionici*. J. Biochem. **83**:191-200.

Kaspar, H. 1982. Nitrite reduction to nitrous oxide by propionibacteria: Detoxification Mechanism. Arch. Microbiol. **133**:126-130.

Kauc. L., M. Mitchell, and S. Goodgal. 1989. Size and physical map of the chromosome of *Haemophilus influenzae*. J. Bacteriol. **171**:2474-2479.

Kemp, A., J. Geurink, R. Haalstra, and A. Malestein. 1976. Nitrate poisoning in cattle. 1. Discolouration of the vaginal mucous membrane as aid in the prevention of nitrate poisoning in cattle. Stikstof. **19**:40-48.

Kemp, A., J. Geurink, Haalstra,and A. Malestein. 1977. Nitrate poisoning in cattle. 2. Changes in nitrite in rumen fluid and methemoglobin formation after high nitrate intake. Neth. J. agric. Sci. **25**:51-62.

Kemp, A., J. Geurink, A. Malestein, and A. van't Klooster. 1978. Grassland production and nitrate poisoning in cattle. Proc. 7th gen. Meet. Eur. Grassld. Fedn. (Gent).

Ketchum, P., G. Denariaz, J. LeGall, and W. Payne. 1991. Menaquinol-nitrate oxidoreductase of *Bacillus halodenitrificans*. J. Bacteriol. **173**:2498-2505.

Kilgore, G. 1993. Plants & growing conditions which are susceptible to high nitrate concentrations. In: Management of nitrate forages for beef and dairy cattle, G. Highfill (Ed.). Okla. Coop. Ext. Serv., Enid.

Knowles, R. 1982. Denitrification. Microbiol. Rev. **46**:43-70.

Kornyeva, V. 1981. Establishment of propionic acid bacteria in the intestine of infants given propioni-acidophilus milk. Ref. Dairy Sci. Abstr. 1982, **44**:420.

Korzeniowski, A., J. Geurink, and A. Kemp. 1980. Nitrate poisoning in cattle. 5. The effect of tungsten on nitrite formation by rumen microbes. Neth. J. agric. Sci. **28**:16-19.

Korzeniowski, A., J. Geurink, and A. Kemp. 1981. Nitrate poisoning in cattle. 6. Tungsten (wolfram) as a prophylatic against nitrate-nitrite intoxication in ruminants. Neth. J. agric. Sci. **29**:37-47.

Ladror, U., L. Gollapudi, R. Tripathi, S. Latshaw, and P. Kemp. 1991. Cloning, sequencing, and expression of pyrophosphate-dependent phosphofructokinase from *Propionibacterium freudenreichii*. J. Biol. Chem. **266**:16550-16555.

Lam, Y. and D. Nicholas. 1969. A nitrate reductase from *Micrococcus denitrificans*. Biochim. Biophys. Acta. **178**:225-234.

Lambden, P., and J. Guest. 1976. Mutants of *Escherichia coli* K12 unable to use formate as an anaerobic electron acceptor. J. Gen. Microbiol. **97**:145-160.

Langsrud, T., and G. Reinbold. 1973. Flavor development and mcirobiology of Swiss cheese-a review. II. Starters, manufacturing processes and procedures. J. Milk Food Technol. **36**:531-542.

Langsrud, T., and G. Reinbold. 1973. Flavor development and microbiology of Swiss cheese a review. IV. Defects. J. Milk Food Technol. **37**:24-41.

Lester, R., and J. DeMoss. 1971. Effects of molybdate and selenite on formate and nitrate metabolism in *Escherichia coli*. J. Bacteriol. **105**:1006-1014.

Li, S., and J. DeMoss. 1987. Promoter region of the *nar* operon of *Escherichia coli*: nucleotide sequence and transcription initiation signals. J. Bacteriol. **169**:4614-4620.

Li, S., and J. DeMoss. 1988. Location of sequences in the *nar* promoter of *Escherichia coli* required for regulation by Fnr and NarL. J. Biol. Chem. **263**:13700-13705.

Li, S., T. Rabi, and J. DeMoss. 1985. Delineation of two distinct regulatory domains in the 5' region of the *nar* operon of *Escherichia coli*. J. Bacteriol. **164**:25-32.

Lyon, W., and B. Glatz. 1991. Partial purification and characterization of a bacteriocin produced by *Propionibacterium theonii*. Appl. Environ. Microbiol. **57**:701-706.

MacGregor, C., C. Schnaitman, D. Normansell, and M. Hodgins. 1974. Purification and properties of nitrate reductase from *Escherichia coli* K12. J. Biol. Chem. **249**:5321-5327.

MacGregor, C. 1975a. Solubilization of *Escherichia coli* nitrate reductase by a membrane-bound protease. J. Bacteriol. **121**:1102-1110.

MacGregor, C. 1975b. Anaerobic cytochrome b1 in *Escherichia coli*: association with and regulation of nitrate reductase. J. Bacteriol. **121**:1111-1116

MacGregor, C. 1975c. Synthesis of nitrate reductase components in chlorate-resistant mutants of *Escherichia coli*. J. Bacteriol. **121**:1117-1121.

MacGregor, C., and A. Christopher. 1978. Asymmetric distribution of nitrate reductase subunits in the cytoplasmic membrane of *E. coli*: evidence derived from surface labeling studies with transglutaminase. Arch. Biochem. Biophys. **185**:204-213.
MacGregor, C., and G. McElhaney. 1981. New mechanism for post-translational processing during assembly of a cytoplamic membrane protein? J. Bacteriol. **148**:551-558.

Malestein, A., J. Geurink, G. Schuyt, A. Schotman, A. Kemp,and A. van't Klooster. 1980. Nitrate poisoning in cattle. 4. The effect of nitrite dosing during parturition on the oxygen capacity of maternal blood and the oxygen suppley to the unborn calf. Vet. Quart. **2**:149-159.

Maniatis, T., E. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Mantere-Alhonen, S. 1982. Die Propionibakterien der Molkereiindustrie als Darmkanalmikroben. Meijeritieteellinen Aikakauskirja. **XL** (1) 95 S.

Mantere-Alhonen, S. 1983. On the survival of a *Propionibacterium freudenreichii*-culture during 'in vitro' gastric digestion. Meijeritieteellinen Aikakauskirja. **XLI:**19-23.

Mantere-Alhonen, S., and E. Makinen. 1987. A new type of sour milk with propionibacteria. Meijeritieteellinen Aikakauskirja. XLV:49-61.

Mantere-Alhonen, S., and H. Mllymaki. 1985. A substrate for the production of propionibacteriamass. Kemia-Kemi short communications, n:o 11.

Marwha, S., J. Kennedy, and R. Sethi. 1983. Vitamin B_{12} production from whey and simulation of optimal cultural conditions. Process. Biochem. **18**:24-27.

McPherson, M., A. Baron, D. Pappin, and J. Wootton. 1984. Respiratory nitrate reductase of *Escherichia coli*. Sequence identification of the large subunit gene. FEBS Lett. 177:260-264.

Meyers, J., D. Sanchez, L. Elwell, and S. Falkow. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. **127**:1529-1537.

Murakami, K., Y. Hashimoto, and Y. Murooka. 1993. Cloning characterization of the gene encoding glutamate 1-semialdehyde 2,1-aminomutase, which is involved in δ-Aminolevulinic acid synthesis in *Propionibacterium freudenreichii.* Appl. Environ. Microbiol. **59**:347-350

Nabukhotnyi, T., S. Cherevko, G. Sanigullina, and A. Grushko. 1985. Use of adapted propioni-acidophilic "Malyutka" and "Malyush" formulas in complex treatment of acute gastrointestinal diseases of infants. Ref. Dairy Sci. Abstr. 1985, **47**:2122.

Nohno, T., S. Noji, S. Taniguchi, and T. Saito. 1989. The *narX* and *narL* genes encoding the nitrate-sensing regulators of *Escherichia coli* are homologous to a family of prokaryotic two-component regulatory genes. Nucleic Acids Res. **17**:2947-2957.

Noji, S., T. Nohno, T. Saito, and S. Taniguchi. 1989. The *narK* gene product participates in nitrate transport induced in *Escherichia coli* nitrate-respiring cells. FEBS. **252**:139-143.

Park, H., G. Reinbold, and E. Hammond. 1967. Role of propionibacteria in split defect of Swiss cheese. J. Dairy Sci. **50**:820-823.

Perez-Mendoza, J. and F. Garcia Hernandez. 1983. Fermentation of a waste-product from the industrial processing of the lime (Citrus aurantifolia swingle) for vitamin B_{12} production. I. Culture conditions for *Propionibacterium shermanii* and effect of 5,6-dimethylbenzimidazole as precursor. Biotechnol. Lett. **5**:259-264.

Perlman, D. 1978. p.303-326. In: Rose, A. H. (ed.) Economic microbiology, vol. 2., Primary products of metabolism. Academic Press Inc., London.

Playne, M. 1985. p.731-759. Propionic and butyric acids. In: Moo-Young, M. (ed.) Comprehensive biotechnology. Pergamon Press, Oxford.

Rabin, R., L. Collins,and V. Stewart. 1992. In vivo requirement of intergration host factor for nar (nitate reductase) operon expression in *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA. **89**:8701-8705.

Rabin, R., and V. Stewart. 1992. Either of two functionally redundant sensor proteins, *narX* and *narQ* is sufficient for nitrate regulation in *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA. **89**:8419-8423.

Rabin, R. ,and V. Stewart. 1993. Dual response regulators (*narL* and *narP*) interact with dual sensors (*narX* and *narQ*) to control nitrate- and nitrite- regulated gene expression in *Escherichia coli* K-12. J. Bacteriol. **175**:3259-3268.

Radcliffe, B. and D. Nicholas. 1970. Some properties of a nitrate reductase from *Pseudomonas denitrificans*. Biochim. Biophys. Acta. **205**:273-287.

Raeker, M., C., Bern, L. Johnson and B. Glatz. 1992. Preservation of high-moisture maize by various propionate treatments. Cereal Chem. **69**:66-69.

Rehberger, T. 1984. Thesis. Iowa State University

Rehberger, T. 1993. Genome analysis of *Propionibacterium freudenreichii* by pulsed-field electrophoresis. Curr. Microbiol. **27**:21-25.

Rehberger, T., C. Hibberd, and J. Swartzlander. 1993. p.E1-E15. Identification and application of a propionibacteria strain for nitrate and nitrite reduction in the rumen. In: Management of high nitrate forages for beef and dairy cattle, G. Highfill (Ed.) Okla. Coop. Ext. Serv., Enid.

Riet, J. van't (1970). Ph.D. Thesis: Free University, Amsterdam.

Riet, J. van't, A. Stouthamer, and R. Planta. 1968. Regulation of nitrate assimilation and nitrate respiration in *Aerobacter aerogenes*. J. Bacteriol. **96**:1455-1464.

Riet, J. van't, and R. Planta. 1969. Purification and some properties of the membrane-bound respiratory nitrate reductase of *Aerobacter aerogenes*. FEBS. **5**:249-252.

Riet, J. van't, J. van Ed, R. Wever, B. van Gelder, and R. Planta. 1975. Characterization of the respiratory nitrate reductase of *Klebsiella aerogenes* as a molybdenum-containing iron-sulfur enzyme. Biochim. Biophys. Acta. **405**:306-317.

Ronson, C., B. Nixon, and F. Ausubel. 1987. Conserved domains in bacterial regulatory proteins that respond to environmental stimuli. Cell **49**:579-581.

Ruiz-Herrera, J., and J. DeMoss. 1969. Nitrate reductase complex of *Escherichia coli* K-12: isolation and characterization of mutants unable to reduce nitrate. J. Bacteriol. **97**:1291-1297.

Ruiz-Herrera, J., and J. DeMoss. 1969. Nitrate reductase complex of *Escherichia coli* K12: participation of specific formate dehydrogenase and cytochrome b1 component in nitrate reduction. J. Bacteriol. **99**:720-729.

Sanger, F., S. Nicklen, A. Coulson. 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA. **74**:1342-1346.

Sanigullina, F. L, and W. Pawliuk. 1984. Eine experimentelle beurteilung der biologishchen wertigkeit der Propionsaure Acidophilus-Nahrmittel "Malyutka" und "Malyush". Ref. Mi. 1985, 585.

Schulp, J. and A. Stouthamer. 1970. The influence of oxygen, glucose and nitrate upon the formation of nitrate reductase and the respiratory system in *Bacillus licheniformis*. J. Gen. Microbiol. **64**:195-203.

Schulp, J. 1972. Ph.D. Thesis: Free University, Amsterdam.

Shaw, D., and J. Guest. 1983. Homology between CAP and Fnr, a regulator of anaerobic respiration in *Escherichia coli*. J. Mol. Biol. **166**:241-247.

Showe, M., and J. DeMoss. 1968. Localization and regulation of synthesis of nitrate reductase in *Escherichia coli*. J. Bacteriol. **95**:1305-1313.

Sidorchuk, L, and V. Bondarenko. 1984. Selection of a biologically active mutant of *Propionibacterium shermanii* and the possibility of its use in complex therapy of enteral dysbacteriosis. J. Hyg. Epidemiol. Microbiol. Immunol. **28**:331-333.

Sidorchuk, L, V. Bondareko, B. Patraty, S. Cherevko, and T. Nabukhotny. 1984. Efficiency of biopreparation from *Propionibacterium shermanii* in prophylaxis of dysbacteriosis in salmonellosis and shigellosis treatment. Mikrobiol. ZH. **46**:43-46.

Simon, J., J. Sund, M. Wright, A. Winter, and F. Douglas. 1958. Pathological changes associated with the lowland abortion syndrome in Wisconsin. J. Am. Vet. Med. Assoc. **132**:164.

Simoni, R., and M. Schallenberger. 1972. Coupling of energy to active transport of amino acids in *Escherichia coli*. Proc. Natl. Acad. Sci. USA. **69**:2663-2667.

Sinclair, P., and D. White. 1970. Effect of nitrate, fumarate, and oxygen on the formation of the membrane-bound electron transport system of *Haemophilus parainfluenzae*. J. Bacteriol. **101**:365-372.

Sodergren, E., and J. DeMoss. 1988. *narI* region of the *Escherichia coli* nitrate reductase (*nar*) operon contains two genes. J. Bacteriol. **170**:1721-1729.

Somkuti, G., and T. Johnson. 1990. Cholesterol uptake by *Propionibacterium freudenreichii*. Curr. Microbiol. **20**:305-309.

Sone, N. (1972) The redox reactions in propionic acid fermentations. Occurrence and nature of an electron transfer system in *Propionibacterium arabinosum*. J. Biochem. **71**:931-940.

Southern, E. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503-517.

Spiro, S. and J. Guest. (1990). FNR and its role in oxygen-regulated gene expression in *Escherichia coli*. FEMS Microbiol. Lett. **75**:399-428.

Stewart, V. 1982. Requirement of Fnr and NarL functions for nitrate reductase expression in *Escherichia coli* K-12. J. Bacteriol. **151**:1320-1325.

Stewart, V. 1988. Nitrate respiration in relation faculative metabolism in *Enterobacteriaceae*. Microbiol. Rev. **52**:190-232.

Stewart, V., and C. MacGregor. 1982. Nitrate reductase in *Escherichia coli* K12: involvement of *chlC*, *chlE*, and *chlG* loci. J. Bacteriol. **151**:788-799.

Stewart, V., and J. Parales, Jr. 1988. Identification and expression of genes *narL* and *narX* of the *nar* (nitrate reductase) locus in *Escherichia coli* K-12. J. Bacteriol. **170**:1589-1597.

Stewart, V., J. Parales, Jr., and S. Merkel. 1989. Structure of genes *narL* and *narX* of the *nar* (nitrate reductase) locus in *Escherichia coli* K-12. J. Bacteriol. **171**:2229-2234.

Stockbine, N., M. Jackson, L. Sung, R. Holmes, A. O'Brien. 1988. Cloning and sequencing of the genes for shiga toxin from *Shigella dysenteriae* type 1. J. of Bacteriol. **170**:1116-1122.

Stouthamer, A. 1967. Mutant strains of Aerobacter aerogenes which require both methionine and lysine for aerobic growth. J. Gen. Microbiol. **46**:389-398.

Stouthamer, A. 1976. Biochemistry and Genetics of Nitrate Reductase in Bacteria. Adv. Microb. Physiol. **14**:315-375.

Tabib, Z., W. Hagler, and P. Hamilton. 1982. Comparison of physiological parameters useful for assessment of activity of antifungal agents in feed and ingredients. J. Food Prot. **45**:1030-1037.

Tomes, N. 1991. Bacterial treatment ot preserve silage. US Patent 4,981,705.

Tuikov, R. M. Khristova, K. Rizanvov, and I. Vladimirov. 1980. Early weaning of calves using a milk additive and a preparation of freeze-dried bacteria. Dairy Sci. Abstr. 1982, 45 (4):1689.

Vladimirov, I., K. Rizanvov, A. Vasilev, and P. Kenov. 1977. Tests on replacement of antibiotics in sucking calf feeding by a freeze-dried preparation of bacteria. Dairy Sci. Abstr. 1978. **40** (4):2118.

Weiner, J., D. MacIsaac, R. Bishop, and P. Bilous. 1988. Purification and properties of *Escherichia coli* dimethylsulfoxide reductase, an iron-sulfer molybdoenzyme with broad substrate specificity. J. Bacteriol. **170**:1505-1511.

Wimpenny, J., and J. Cole. 1967. The regulation of metabolism in faculative bacteria. III. The effect of nitrate. Biochim. Biophys. Acta. **148**:233-242..

Wimpenny, J. 1969. Oxygen and carbon dioxide as regulators of microbial growth and metabolism. Symposium of the Society for General Microbiology. **19**:161-197.

Woolford, M. 1975. The significance of *Propionibacterium* species and *Micrococcus lactilyticus* to the ensiling process. J. Appl. Bacteriol. **39**:301-306.

Yongsmith, B., and P. Apiraktivongse. 1983. Vitamin B_{12} production from soybean curd whey with *Propionibacterium freudenreichii*. J. Ferment. Technol. **61**:105-107.

VITA 2

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