CHARACTERIZATION OF THE *AROA* GENE OF *BRUCELLA ABORTUS* AND CONSTRUCTION OF AN *AROA* MUTANT

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NOMENCLATURE

amp	Ampicillin
aroA	Gene Encoding EPSP Synthase
bp	Base Pair
cam	Chloramphenicol
DNA	Deoxyribonucleic Acid
EPSP	5-enolpyruvylshikimate 3-phosphate
EtBr	Ethidium Bromide
EtOH	Ethanol
hr	Hour
IPTG	Isopropyl-β-d-Thiogalactopyranoside
kan	Kanamycin
kb	Kilo-base(s)
kDa	Kilo-dalton
kv	Kilo-volt(s)
LB	Luria-Bertani (medium)
mg	Mili-gram
ml	Mili-liter
msec	Mili-second
nt	Nucleotide (s)
ORF	Open Reading Frame
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
TB	Terrific Broth (medium)
X-Gal	5-Bromo-4-chloro-3-Indolyl-β-D-Galactoside

Chapter I

Introduction

Pathogenesis of Brucella abortus

Brucella abortus is a gram-negative zoonotic pathogen which causes abortion and infertility in cattle and a condition known as undulant fever in humans (Nicoletti,1989). Brucella infection in food animals is of particular concern because of the associated economic losses to livestock producers and the potential spread of disease to humans (Acha, 1980). Survival and replication in most phagocytes, particularly macrophages, are critical to the pathogenesis of Brucella infections (Enright, 1990). Intracellular pathogens spend most of their life inside various types of cells, away from serum, and out of contact with circulating antibody. In most studies on these intracellular bacteria, vaccines obtained from living, attenuated strains give best protection because a live vaccine lives long enough in cells to have enough contact with T cells to provide good immunity on subsequent challenges. By contrast, a killed preparation may simply be eliminated too fast to stimulate immune reaction (Eisenstein, T. K.,1983 and Montaraz, J. A.,1986).

Control of Brucella abortus in the U.S.

Control of *B.abortus* infection in cattle is usually done by vaccination with the attenuated *B.abortus* strain B19 (Sangari, 1994). This vaccine was obtained by spontaneous mutation after serial subculture of a pathogenic *B.abortus* strain under

laboratory conditions (Graves, 1943). B19 differs from wild type *B. abortus* strain in its sensitivity to erythritol. a four carbon polyalcohol which is utilized by all Brucella isolates with the exception of *B.ovis* and some strains of *B.canis* (Corbel, 1984). F. J. Sangari reported recently that B19 carries a deletion in the erythritol catabolic genes (Sangari, 1994). Although this vaccine has proven to be an important component of effective eradication and control programs (Acha, 1980), it has several disadvantages such as its virulence for humans (Spink, 1962), the occasional induction of abortion in pregnant animals vaccinated with it and the development of agglutinating antibodies indistinguishable from those elicited by natural infection in animals immunized as adults (Nicoletti, 1990). Brucellosis vaccines lacking these limitations would be of great utility for both veterinary and human medicine.

Development of improved vaccines and potential of an aroA mutant as a living vaccine candidate

It was first reported by Bacon et al. in 1950 (Bacon, 1950) that attenuated auxotrophs of *Salmonella typhi* defective in the aromatic amino acid biosynthetic pathway were avirulent in mice. Subsequently, it has been demonstrated in widely diverse bacteria that disrupting the aromatic amino acid biosynthetic pathway produces attenuated organisms. For example, attenuated strains of the invasive bacteria *S. typhimurium* (Hoiseth, 1981), *Shigella flexneri* (Verma, 1991), and *Yersinia enterocolitica* (Bowe, 1989) were generated by introducing mutations in their respective *aroA* genes. Also, attenuation was produced in the noninvasive bacteria *Bordetella*

pertussis (Roberts. 1990) and *Pasteurella multocida* (Homchampa. 1992) through *aroA* inactivation.

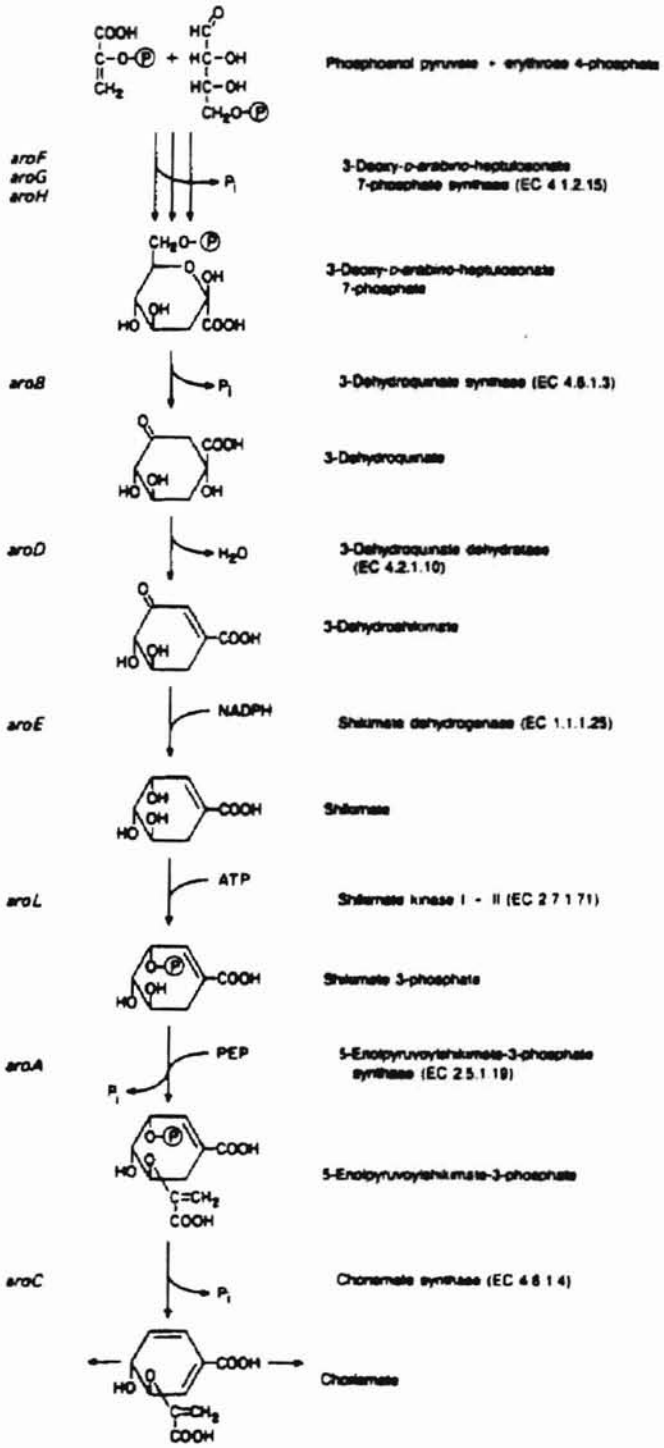
Studies on the aroA gene

The *aroA* gene encodes the enzyme 5-enolpyruvylshikimate 3-phosphate synthase (EPSP synthase; EC 2.5.1.19; alternative name: 3-phosphoshikimate 1-carboxyvinyltransferase) which is the sixth enzyme on the seven - step early common pathway for aromatic amino acid biosynthesis (the pre - chorismate pathway) (Duncan. 1986). Fig.1 shows the common pathway of aromatic acid biosynthesis. The *aroA* mutant strains are unable to synthesize chorismic acid from which p-aminobenzoic acid (PABA) , dihydrobenzoate, and aromatic amino acids are produced. The essential metabolite folate is also synthesized from chorismate via PABA. Most bacteria are not able to assimilate exogenous folate and therefore it must be synthesized from PABA. PABA is not synthesized by humans or other vertebrates as these organisms obtain folate from the diet or from normal flora of the intestine (Griffin. 1995). It is likely that *aroA* mutants of pathogenic bacteria are capable of only limited growth in vertebrate hosts. thus showing attenuated virulence. and so may function as effective oral vaccines.

It was not known whether *B.abortus* could be attenuated by mutation of its *aroA* gene. If atttenuated. such a defined *aroA* mutant may have potential as a live attenuated vaccine against bovine brucellosis. The reported present study showed how we characterized the *aroA* gene of *B.abortus* and attempted to construct an *aroA* mutant strain.

Fig.1 Common pathway of aromatic amino acid biosynthesis

(adapted from K. M. Herrmann and R. L. Somerville, ed., Amino Acids: Biosynthesis and Genetic Regulation, Addison-wesley Publishing Co.)


Phosphoenol pyruvate + erythrose 4-phosphate
aroF
aroG
aroH
3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase (EC 4.1.2.15)
3-Deoxy-D-arabino-heptulosonate 7-phosphate
aroB
3-Dehydroquinate synthase (EC 4.6.1.3)
3-Dehydroquinate
aroD
3-Dehydroquinate dehydratase (EC 4.2.1.10)
3-Dehydroshikimate
aroE
NADPH
Shikimate dehydrogenase (EC 1.1.1.25)
Shikimate
aroL
ATP
Shikimate kinase I - II (EC 2.7.1.71)
Shikimate 3-phosphate
aroA
PEP
5-Enolpyruvoylshikimate-3-phosphate synthase (EC 2.5.1.19)
5-Enolpyruvoylshikimate-3-phosphate
aroC
Chorismate synthase (EC 4.6.1.4)
Chorismate

Chapter II

Materials and Methods

Bacterial strains, plasmids, media and growth conditions

The bacterial strains and plasmids used in this study are listed in table 1. For DNA isolation, *E.coli* strains were routinely cultured in LB (Luria, 1957) or LM plates (Hanahan, 1983) at 37°C overnight. Brucella cells were grown at 37°C under an atmosphere containing 5% CO_2 on plates of tryptose medium (Difco) solidified with 1.5% agarose. For *E.coli* strains, 50 μg ml^{-1} ampicillin and 25 μg ml^{-1} kanamycin were added as required, while 100 μg ml^{-1}, 10 μg ml^{-1} and 25 μg ml^{-1} of ampicillin, kanamycin and chloramphenicol were used respectively for Brucella. Aromatic amino acid requirements were tested using minimal medium or GM medium (Gerhardt and Wilson, 1950) for *E.coli* and *B.abortus* respectively, and, when required, supplemented with 'aromix' which consists of phenylalanine, tryptophan, tyrosine, 4-aminobenzoic acid, 2,3-dihydroxybenzoic acid and 4-hydroxybenzoic acid, all at 10^{-6} M (Pittard and Wallace, 1966).

The *aroA* gene from *B.abortus* 2308 was cloned by complementation of the *E.coli aroA* deficient mutant RE696. Strain RE734, carrying the *aroA* on plasmid pRE167 was chosen for this study.

Preparation and manipulation of DNA

Plasmid DNA was isolated by the alkaline-SDS method (Jones and Schofield, 1990). Genomic DNA from *B.abortus* 2308 was prepared as described in the Current

Protocols in Molecular Biology (Wilson,1994). Restriction endonucleases, exonuclease III, S1 nuclease, Bal31 exonuclease, DNA polymerase I large fragment and T_4 DNA ligase were purchased from Promega or Bethesda Research Laboratory (GibcoBRL) and used under conditions specified by their manufacturers. *E.coli* cells were transformed by the Hanahan (1983) method. Standard techniques were used for molecular cloning and electrophoresis (Ausubel, 1994).

Construction of nested deletions and location of aroA

In order to locate the *aroA* gene in pRE167, a series of unidirectional nested deletions were generated. Deletions from the *Sac*I side were constructed using exonuclease III according to the instructions of the Promega Erase-a-Base system technical manual. Plasmid pRE167 was linearized by *Sac*I plus *Bam*HI, then the single strand of the 5' overhanging end was deleted by ExoIII for various intervals. S1 nuclease was added to digest the remaining single strand, then Klenow fragment and dNTPs were added to repair the ends. The resulting DNA was ligated back to itself with T_4 DNA ligase, then transformed into XL1-blue cells. Plasmid DNA was isolated from randomly picked colonies, and the size was checked by restriction endonuclease digestion and electrophoresis.

Deletions from the *Apa*I side were constructed using Bal31 exonuclease according to the Current Protocols in Molecular Biology (Nixon,1988). Plasmid pRE167 was linearized by *Apa*I, both strands were deleted in both directions by Bal31 for various times, then the reaction was stopped by addition of 200 mM EDTA and heating at 65°C

for 5 minutes. Resulting DNA was digested with *Sac*I, and the insert bands were extracted from low melting agarose by the freezing thaw method (personal communication). Deleted inserts were ligated into pBluescript SK (Stratagene) which was digested with *Sac*I plus *Sma*I, then transformed into XL1-blue and plated on LM with 0.1 M IPTG, 2% X-Gal and 50 μg ml^{-1} amp (TAXI plate). Sizes of plasmids in white colonies were checked as described above.

Nucleotide sequence determination and analysis

The dideoxy method described by Sanger et al (Sanger, 1977) was used to determine the nucleotide sequence of a 2.3 kb region of pRE167 (pRE167A10). The nucleotide sequences of the various deletions were determined by the Oklahoma State University Recombinant DNA/Protein Resource Facility using cycle sequencing and read on an ABI 373A sequencer. Gaps remaining in the sequence were completed by using DNA primers synthesized by the Oklahoma State University Recombinant DNA/Protein Resource Facility. Genetics Computer Group (GCG), oligo 4.0 (National Biosciences, Inc., Plymouth, MN), and Macvector (International Biotechnologies, Inc., New Haven, CT) software packages were used for the analysis of both nucleotide sequences and deduced amino acid sequences. Pileup was used to generate the original alignment which was refined by the Clustal program (w version). This program was used to get the Clustree by the neighbor joining method. Bootstrap program was used to generate 200 independent trees for the Clustree. Different programs in the Phylip package were also used to create phylogenetic trees. Protpars was used to get the Parstree by the parsimony

method; Protdist was used to calculate the distances which were submitted to Fitch to generate the Fitchtree.

Identification of gene products by the maxicell technique

Proteins coded by various plasmids were labeled by the maxicell technique (Sancar, 1981), using strain CSR 603 (Sancar. 1978). Proteins were separated by one-dimensional SDS-PAGE on 12% gels (Laemmli. 1970) or by two-dimensional gel electrophoresis (Ames and Nikaido. 1976). One dimensional gels were loaded with equal amounts of radioactivity in each lane. Gels were soaked in Autofluor (National Diagnostics) and exposed to Kodak XAR-5 film at room temperature. Molecular weight markers and carbamylated carbonic anhydrase were purchased from Sigma Chemical Company, St. Louis, MO, USA. 5 µl of the carbamylated standard was mixed with each sample and run on the two-dimensional gel. After soaking in 0.1% coomassic blue dye solution and washing with 40% methanol/10% acetic acid, carbamylated standard dots showed up and a picture of the gel was taken for later calculation.

Construction of replacement plasmids

The marker-exchange mutagenesis technique was attempted to mutate a specific locus on the chromosome by the homologous exchange recombination of a deletedly or insertionally inactivated, cloned gene carried on an unstable replicon with its functional allele (Ried, 1987). pBluescript was used as the suicide plasmid vector because it is a ColE1-based plasmid, incapable of autonomous replication in Brucella spp. (Halling,

1991 and Rigby, 1989). Four replacement plasmids were constructed. The steps are presented below.

Plasmid pRE167 was partially digested with *Eco*RI and deleted for the 2 kb *Eco*RI fragment by religating the 5.6 kb piece to give pTG101. pTG101 was digested with *Sac*I plus *Apa*I plus *Sca*I to release the 2.7 kb fragment, which carries the deleted *aroA*, then was ligated to pRE257 (Table 1.) digested with *Sac*I plus *Apa*I to make pTG102 (Fig. 2). Ligation was transformed into XL1-blue and plated on TAXI plates. White colonies were picked and the sizes of their inserts were identified by *Eco*RI and *Sac*I plus *Apa*I digestions.

pTG101 was partially digested with *Eco*RI to get a single cut and repaired with Klenow, then ligated with the *Bam*HI digested and Klenow repaired pUM24 (Reid, 1987) to give pTG103 (Fig. 3). Ligation was transformed into XL1-blue and plated on LM with amp and kan. *Pst*I and *Apa*I plus *Eco*RI digestions were done to check sizes.

pTG104 was made by inserting the *Bam*HI cut sac-kan^r cassette which was derived from pUM24 into pRE167 partially digested with *Sau*3A to give a single cut at the middle of the 2 kb *Eco*RI fragment (Fig. 4). The ligation was transformed into RE696, then plated on LM with amp and kan. aro$^-$ colonies were identified by testing them on minimal plates with and without 'aromix'. *Pst*I, *Apa*I plus *Pst*I and *Eco*RI plus *Pst*I digestions were done to check sizes and location of the insert.

pTG105 was made by inserting the *Bam*HI cut sac-kan^r cassette which was derived from pUM24 into the *Sma*I site of pRE167A10 (Fig. 5). Procedures were similar to those used in construction of pTG104.

Introduced replacement plasmids into B.abortus by electroporation

B.abortus 2308 was transformed by electroporation as described by Lai et al (1990) using an ElectroCell Manipulator 600 Electroporation System (Biotechnologies & Experimental Research Inc. San Diego, CA, USA). DNA samples were prepared using the Wizard method (Promega) and eluted with H_2O to a final concentration of 0.5~1 μg μl^{-1}. Cells were harvested from tryptose plates after 2 days growth at 37 °C , 5 % CO_2 and washed with chilled H_20 three times. 0.5 ~1.0 μg plasmid and 60 μl competent cells were transferred to a 0.1 mm gap cuvette and electroporated at 12.5 kv cm^{-1} with R7 resistance, giving a time constant of approximately 11.5 msec. After pulsing, 1ml SOC-B broth (Lai, 1990) was added, cells were incubated at 37°C for 1 hr, then cultured on tryptose plates overnight before plating on selective media.

The strategy of creating a *B.abortus aroA* mutant with one of the replacement plasmids, pTG105 is shown in Fig. 6.

Southern blot analysis

DNA hybridizations were performed using the method of Southern (1975). Probe DNA was labeled with photobiotin and detected by the Southern-Light Chemiluminescent Detection System (Tropix).

TABLE 1. Bacterial strains and plasmids used in this study

Strains	Characteristics	Source or reference
Bacteria		
E.coli K-12		
RE696	F⁻ *aroA354* λ⁻ *supE42 gyrA* Δ(*recA-srlR*)*306 srlR301*:: Tn*10-84*	RCE[a]
RE734	RE696 with pRE167	RCE
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR1 supE44 relA1 lac* [F' *proAB lacI8 ZΔM15* Tn*10* (*Tet*^{r})]	Bullock et al. (1987)
CSR603	*thr-1 leuB6 proA2 phr-1 recA1 argE3 thi-1 uvrA6 ara-14 lacY1galK2 xyl-5 mtl-1 rpsL31 tsx-33 supE44*	Sancar & Rupert. (1978)
B. abortus		
2308		OADDL[b]
Plasmids		
pSK	pBluescript SK⁻, cloning vector (amp^r)	Stratagene
pUM24	with gene sacBR kan in it, a source of sac-kan^r cassette	Ried et al. (1987)
pBBR	positive control plasmid for electroporation (cam^r)	Kovach et al. (1994)
pRE257	pSK with sac-kan^r in amp gene	RCE
pRE167	4.6 kb *Sau*3A fragment containing *B. abortus aroA* in pSK	RCE
pTG101	pRE167 without the 2 kb *Eco*RI fragment	This work
pTG102	2.6 kb pTG101 insert cloned into pRE257	This work
pTG103	sac-kan^r cassette from pUM24 inserted into the *Eco*RI site of pTG101	This work
pTG104	sac-kan^r cassette from pUM24 inserted into pRE167 partially digested with *Sau*3A	This work
pTG105	sac-kan^r cassette inserted into the *Sma*I site of pRE167A10	This work
pRE167A10	pRE167 with 2.3 kb deleted from *Apa*I side	This work

[a] RCE, Richard C. Essenberg

[b] OADDL. Oklahoma Animal Disease Diagnostics Laboratory, OSU, OK.

Fig. 2 Construction of plasmids pTG101 and pTG102.

The restriction enzyme sites are: A, *Apa*I; S, *Sac*I; E, *Eco*RI.

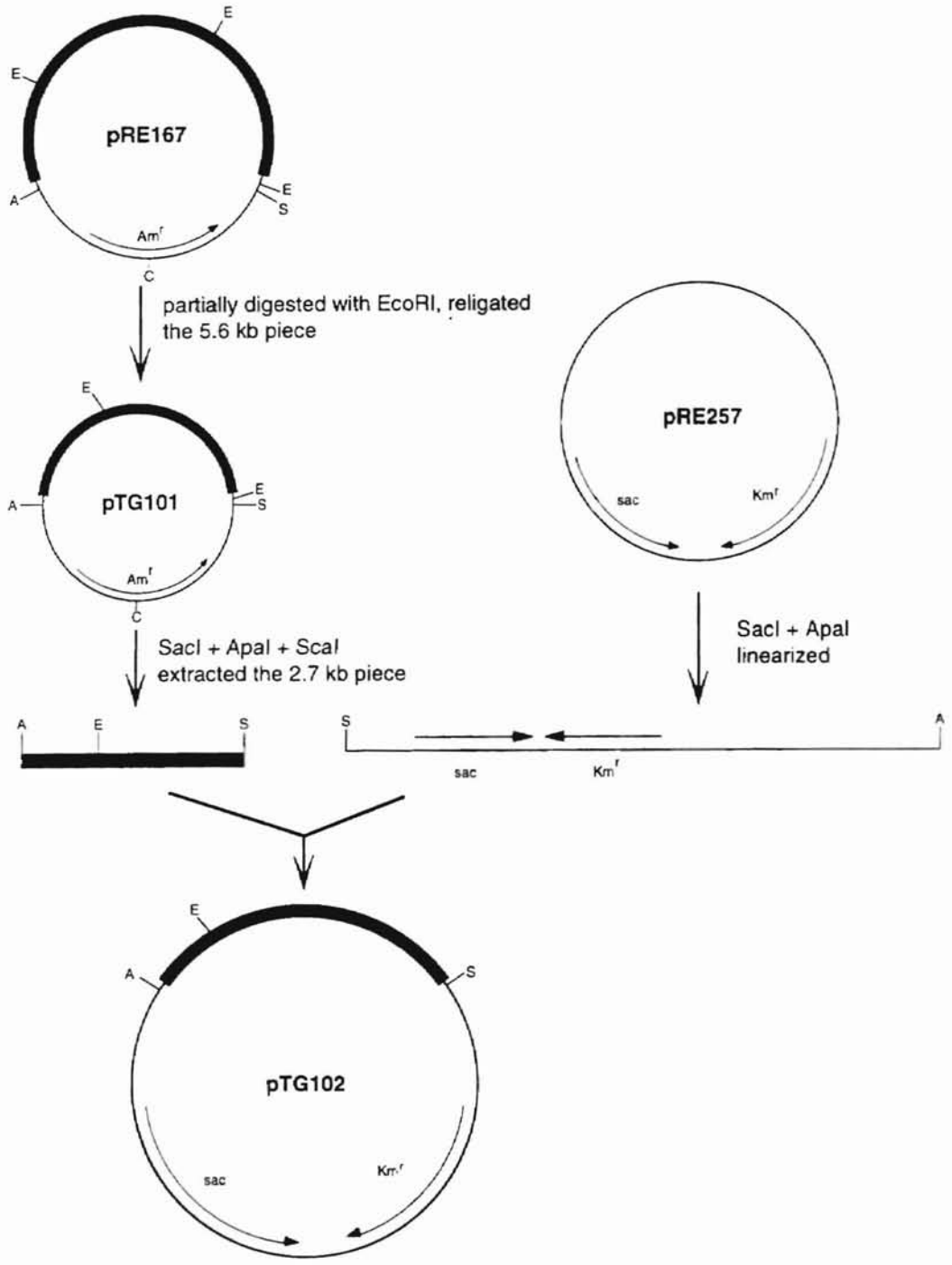

pRE167
E
E
A
E
S
Amr
C
partially digested with EcoRI, religated
the 5.6 kb piece
pTG101
E
A
E
S
Amr
C
SacI + ApaI + ScaI
extracted the 2.7 kb piece
pRE257
sac
Kmr
SacI + ApaI
linearized
A
E
S
S
A
sac
Kmr
pTG102
E
A
S
sac
Kmr

Fig. 3 Construction of replacement plasmid pTG103.

The restriction enzyme sites are: A, *Apa*I; E, *Eco*RI; S, *Sac*I; B, *Bam*HI; P, *Pst*I; C, *Sca*I.

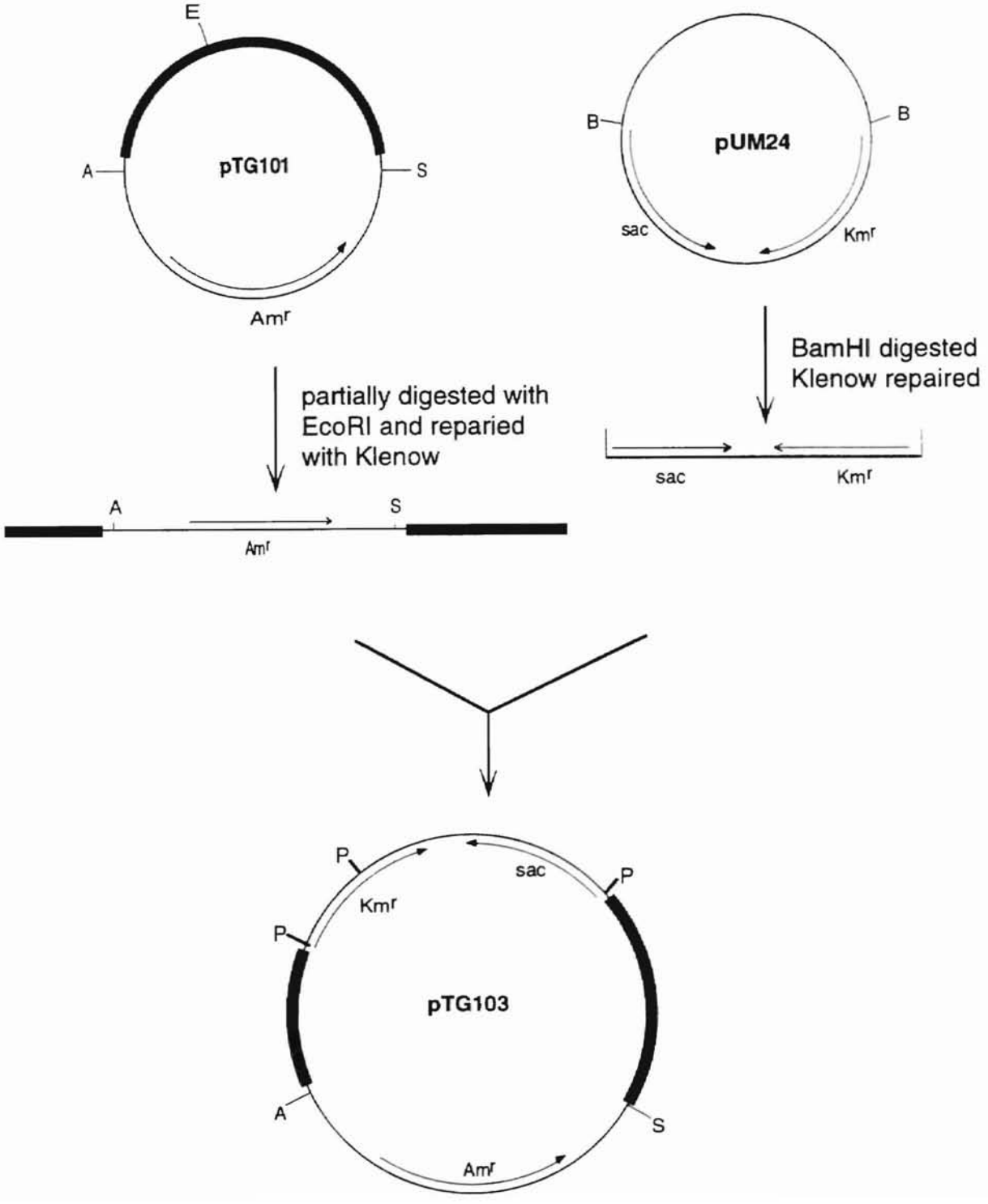

E
pTG101
A
S
Amr
pUM24
B
B
sac
Kmr
BamHI digested
Klenow repaired
sac
Kmr
partially digested with
EcoRI and reparied
with Klenow
A
S
Amr
P
Kmr
sac
P
P
pTG103
A
S
Amr

Fig. 4 Construction of replacement plasmid pTG104.

The restriction enzyme sites are: A, *Apa*I; E, *Eco*RI; S, *Sac*I; B. *Bam*HI.

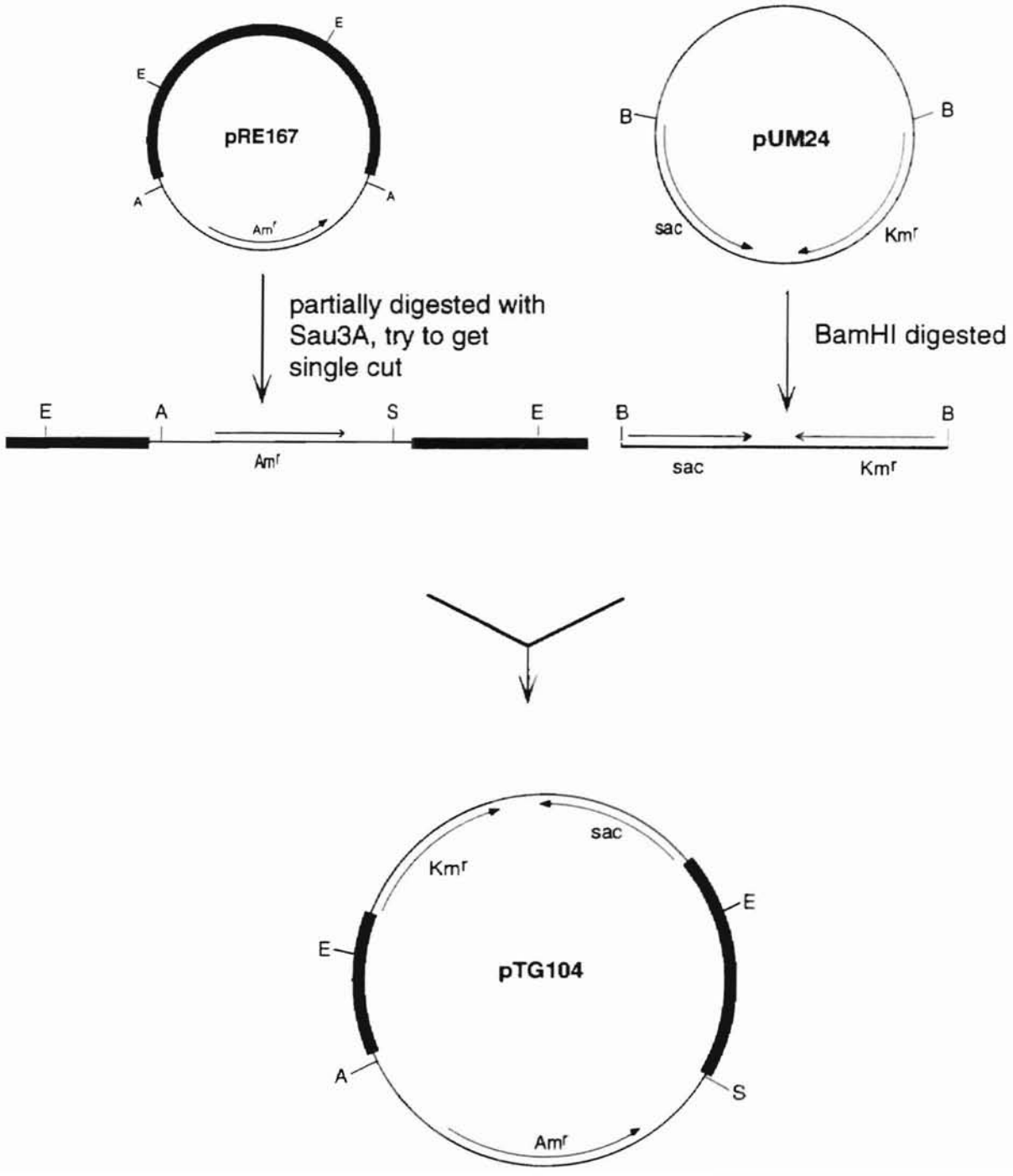

pRE167
E
E
A
A
Amr
partially digested with
Sau3A, try to get
single cut
E
A
S
E
Amr
pUM24
B
B
sac
Kmr
BamHI digested
B
B
sac
Kmr
sac
Kmr
E
E
pTG104
A
S
Amr

Fig. 5 Construction of replacement plasmid pTG105

The restriction enzyme sites are: A, *Apa*I; E, *Eco*RI; S, *Sac*I; B, *Bam*HI; M, *Sma*I.

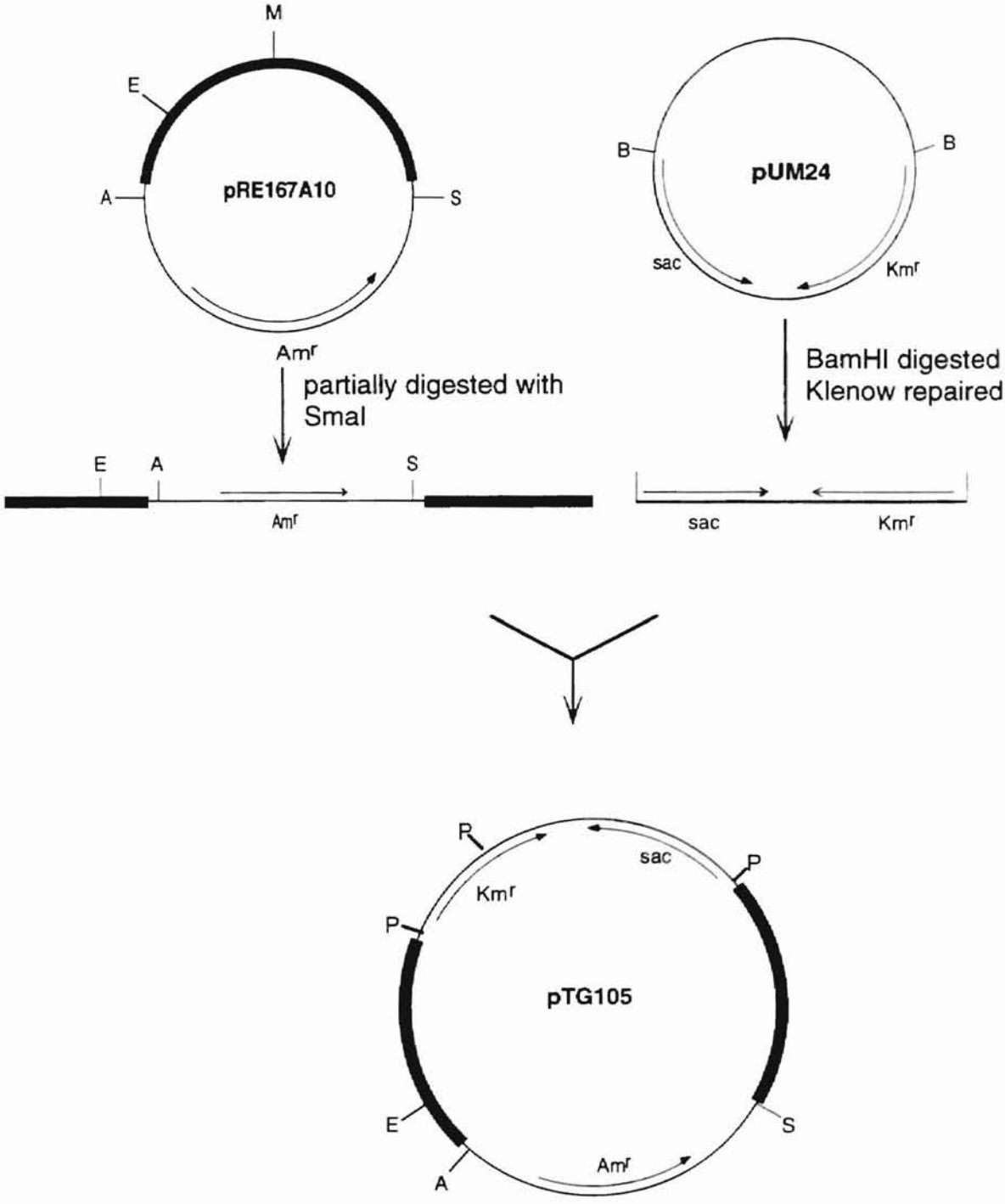
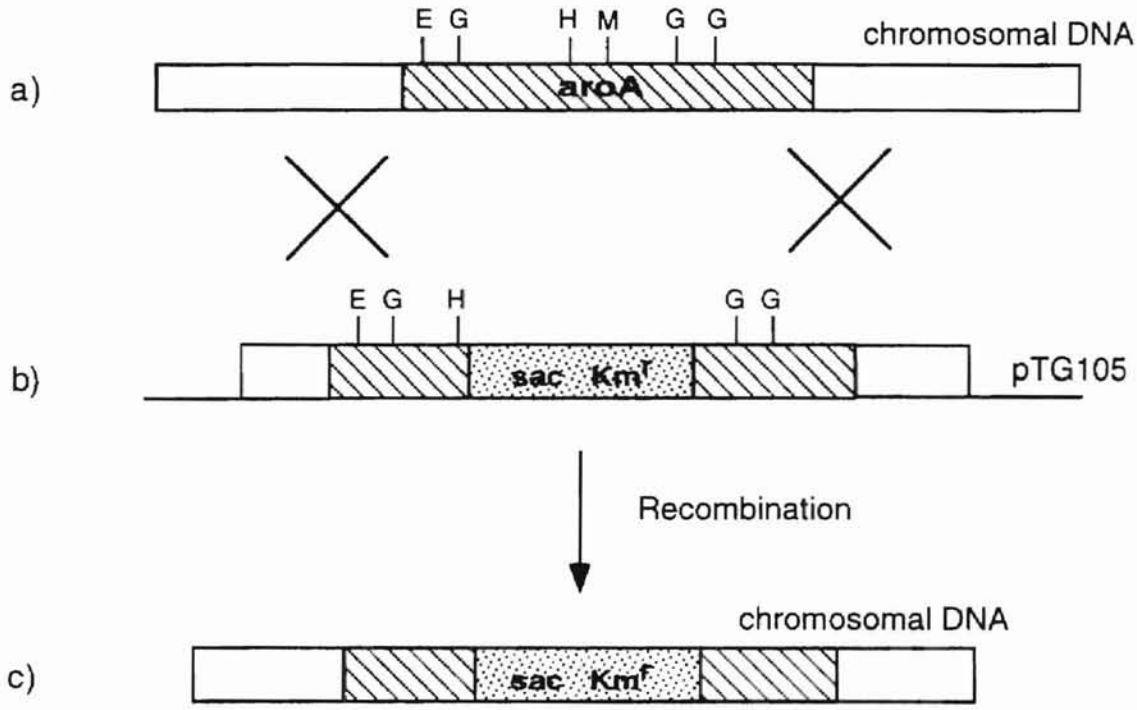

M
E
A
pRE167A10
S
Amr
partially digested with
SmaI
E
A
S
Amr
B
B
pUM24
sac
Kmr
BamHI digested
Klenow repaired
sac
Kmr
P
Kmr
sac
P
P
pTG105
E
S
A
Amr

Fig. 6 Gene replacement strategy used for the creation of a *B. abortus aroA* mutant.

a) The restriction enzyme sites within *aroA* are: E, *Eco*RI; G, *Bgl*I; H, *Hin*dIII; M, *Sma*I.
b) The construction of pTG105 is described in Fig. 5.
c) Integration of the suicide plasmid into the chromosome by a double crossover should give rise to *B. abortus* 2308 kan^r suc^s amp^s $aroA^-$.


a)
E G
H M
G G
chromosomal DNA
aroA
b)
E G
H
G G
sac
Km^r
pTG105
Recombination
c)
chromosomal DNA
sac
Km^r

Chapter III

Results

Nested deletions and location of aroA

Deletion from the *Apa*I side with Bal31 was done three times to get the whole plasmid deleted enough to be sequenced. Fig. 8 shows the first deletion by Bal31 to get subclones pRE167A1 to pRE167A6. From pRE167A6, the same method was used to make smaller subclones pRE167A7 to pRE167A10. Again, pRE167A10 was further deleted as described above. To check the sizes of these subclones, *Apa*I plus *Sac*I digestion was done (Fig. 8). Deletion from the *Sac*I side with ExoIII was done just once to get the whole pRE167 deleted (Fig. 9). Several combinations of restriction enzyme digestions were done to check the sizes of these deletions (Data not shown).

These deletions and various subclones were transformed into strain RE696: deletions from pRE167A101 to pRE167A108 and all the deletions from the *Sac*I side are aro^{-} . One subclone, pRE234, thought to contain the two rightmost *Eco*RI fragments and to be aro^{-} actually contained different fragments. The restriction map and subclones of pRE167 are shown in Fig. 10. On the basis of this map, the *aroA* gene should be in plasmid pRE167A10 and we sequenced this region.

Nucleotide sequence analysis of the aroA gene

The nucleotide sequence of the 2290 bp insert in pRE167A10 was determined by automated methods (Fig. 11) and revealed two open reading frames. The bigger one is in the expected position of *aroA*, has 1440 bp with a coding capacity of 480 amino acid

residues. The deduced molecular weight is 51 kDa and pI is 5.24. The small ORF following the *aroA* is 558 bp with a coding capacity of 186 amino acid residues, coding for a 19 kDa, pI 9.26 protein which showed 60.75% and 56.83% similarity to the cytidylate kinase of *Synechocystis* sp. and *E.coli* respectively. Alignment of the deduced amino acid sequences encoded by *aroA* genes from different bacteria is shown in Fig. 12.

Phylogenetic trees developed by the neighbor joining, Fitch , and parsimony methods are shown in Fig.13.

Identification of proteins coded by the clones

Three major proteins were seen for pRE167 in maxicell experiments (Fig. 14 and 15), one of which, also seen with pBluescript, had the molecular weight expected for β-lactamase (33 kDa). Another fainter band appeared above this band with an apparent molecular weight of 53 kDa , but was absent from a deletion without *aroA* (pRE167A103) or shortened for a deletion from the *Sac*I side (pS0403). The third band had an apparent molecular weight of 72 kDa and was absent from deletions from the *Apa*I side (pRE167A9, pRE167A10 and pRE167A103). The same result was seen for the two-dimensional gel of pRE167 and pRE167A103 (Fig.15): the 72 kDa and 53 kDa protein showed in pRE167 but not in pRE167A103. The 72 kDa protein is acidic, the 53 kDa one is neutral. However, we couldn't find the 19 kDa protein expected from the second ORF of pRE167.

Construction of replacement plasmids and introduction into B.abortus by electroporation

Four replacement plasmids were constructed. The first three of them were constructed based on the original restriction map of pRE167 which has the pRE234 error in it. According to this map, the 2 kb *Eco*RI fragment is essential for the *aroA*. We attempted to use the 2 kb *Eco*RI fragment deletion plasmid, pTG102, carrying the sac-kan^r in the cloning vector as a suicide plasmid to get a mutation without introducing any drug resistance. This strategy was described by Ried et al (Ried, 1987): selection for kan resistance gives single crossover insertions that have a duplication of the target gene *aroA* with one good and one mutant copy from homologous recombination. In the second step, excision of one copy of the *aroA* gene and the vector is selected for by the *sacB* gene, whose presence makes the cells sensitive to sucrose. The replacement possibility is about 50% because the probability of eliminating the wild type or mutant gene is roughly equal. After several attempts to introduce the construct into *B.abortus* by electroporation, only one colony showed kan resistance and sucrose sensitivity. It was cultured on tryptose plates for two days and different dilutions of cells were plated on tryptose with 5% sucrose. Single colonies were picked from sucrose plates and tested for sensitivity to kan. Most of them were kan sensitive. These kan^s sucrose^r colonies were tested on GM plates with and without 'aromix'. None of them showed aro$^-$. Genomic DNA was isolated from six of these colonies, then digested with *Eco*RI and analyzed by Southern blotting. The 2 kb *Eco*RI fragment was not deleted for any of the six colonies (data not shown).

Because of the lack of success of the two step selection strategy, we tried to replaced the 2 kb *Eco*RI fragment with the sac-kan^r and hoped we could get a mutant by one step selection. No matter whether a single crossover or double crossover event happens, it should give kan^r sucrose^s, but a single crossover should give amp^r while a double crossover should not. pTG103 was transformed into *B.abortus*, but all the kan^r colonies were amp^s and no sucrose^s colony was found.

Since *Eco*RI deletion plasmids pTG102 and pTG103 didn't work, we suspected that the deletion is lethal. We constructed pTG104, which has the sac-kan^r inserted into the undeleted *aro*A gene. Also, no sucrose sensitive or amp^r colonies were found.

We didn't know the exact location of *aroA* until we got the 2.3 kb pRE167A10 sequenced. *aroA* started about 200 kb from the *Sac*I side, ended just a few bases after the *Eco*RI site, so we constructed pTG105 with the sac-kan^r inserted into the *Sma*I site of the *aroA* gene (Fig. 16A and 16B). Still, we didn't get sucrose sensitive colonies. All kan resistant colonies were amp^s.

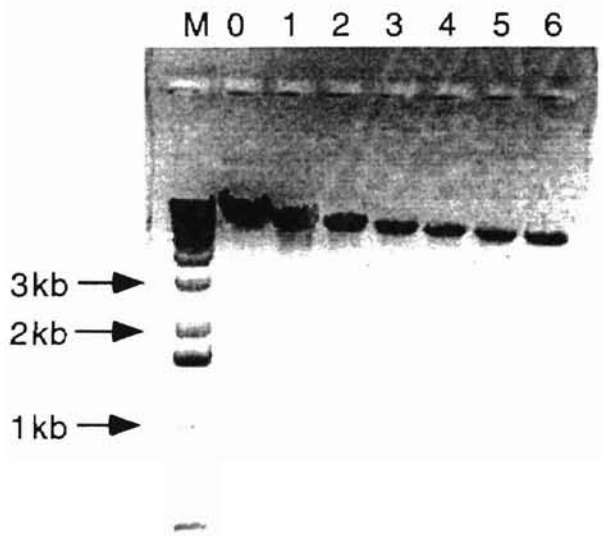
Plasmid pBBR (Kovach, 1994) was used as a positive control because it can replicate in *B.abortus* and a lot of cam resistant colonies showed up. Negative control was done by electroporating *B.abortus* competent cells without any DNA under the same condition, a few kan resistant colonies showed up after 5-6 days incubation.

Fig. 7 Generation of the unidirectional deletions (Bal31 library).

A. Monitoring the progressive Bal31 digestion reaction. Samples were withdrawn at 10 minute intervals. The left lane is 1 kb DNA ladder (BRL). Lane 0 is *Apa*I linearized pRE167 DNA.

B. *Sac*I digestion of pRE167 Bal31 libraries. Lane M, 1 kb DNA ladder. Lane assignments were the same as in (A).

A.



B.

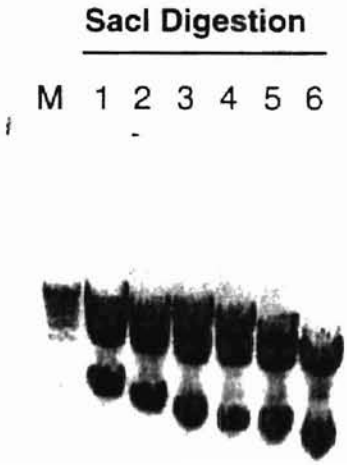
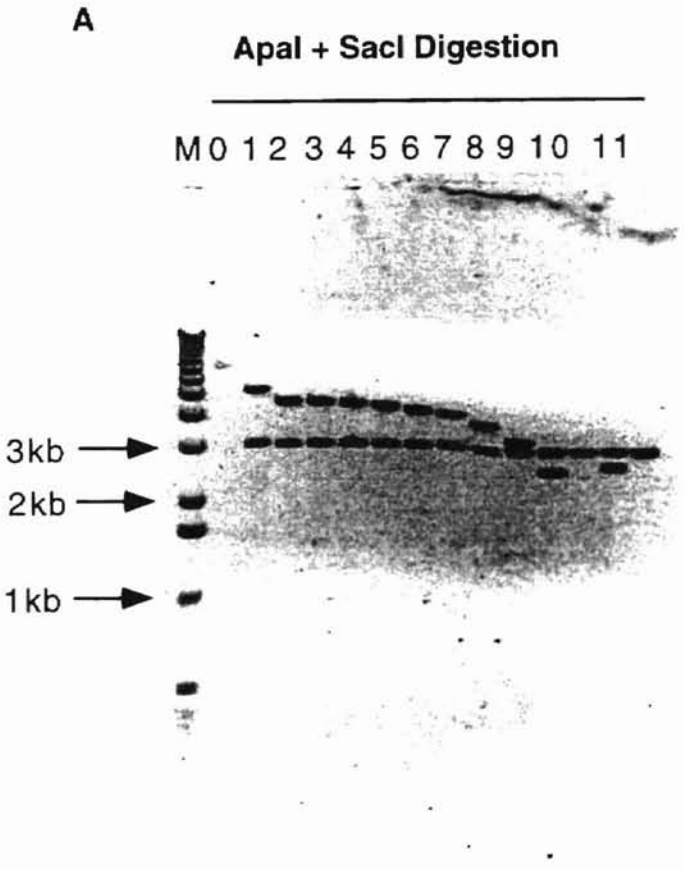


Fig. 8 pRE167 *Apa*I side deletions confirmed by *Apa*I plus *Sac*I digestion.

A. M, 1 kb DNA ladder. Lane 0, uncut pRE167. Lane 1 to lane 11 are: pRE167, pRE167A1, pRE167A2, pRE167A3, pRE167A4, pRE167A5, pRE167A6, pRE167A7, pRE167A8, pRE167A10, pRE167A9.

B. M, 1 kb DNA ladder. Lane 1 to lane 9 are: pRE167A10, pRE167A101, pRE167A102, pRE167A103, pRE167A104, pRE167A105, pRE167A106, pRE167A107, pRE167A108.


A
ApaI + SacI Digestion
M 0 1 2 3 4 5 6 7 8 9 10 11
3kb
2kb
1kb

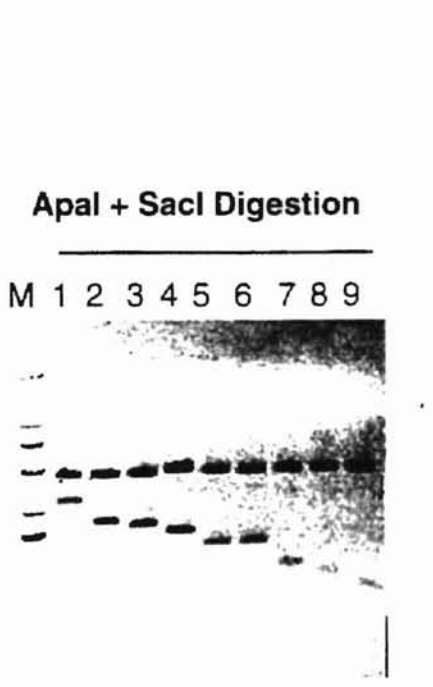

B
ApaI + SacI Digestion
M 1 2 3 4 5 6 7 8 9

Fig. 9 Generation of the unidirectional deletions (ExoIII library)

Monitoring the progressive Exonuclease III digestion reaction. Samples were withdrawn at 30 second intervals. M, 1 kb DNA ladder.

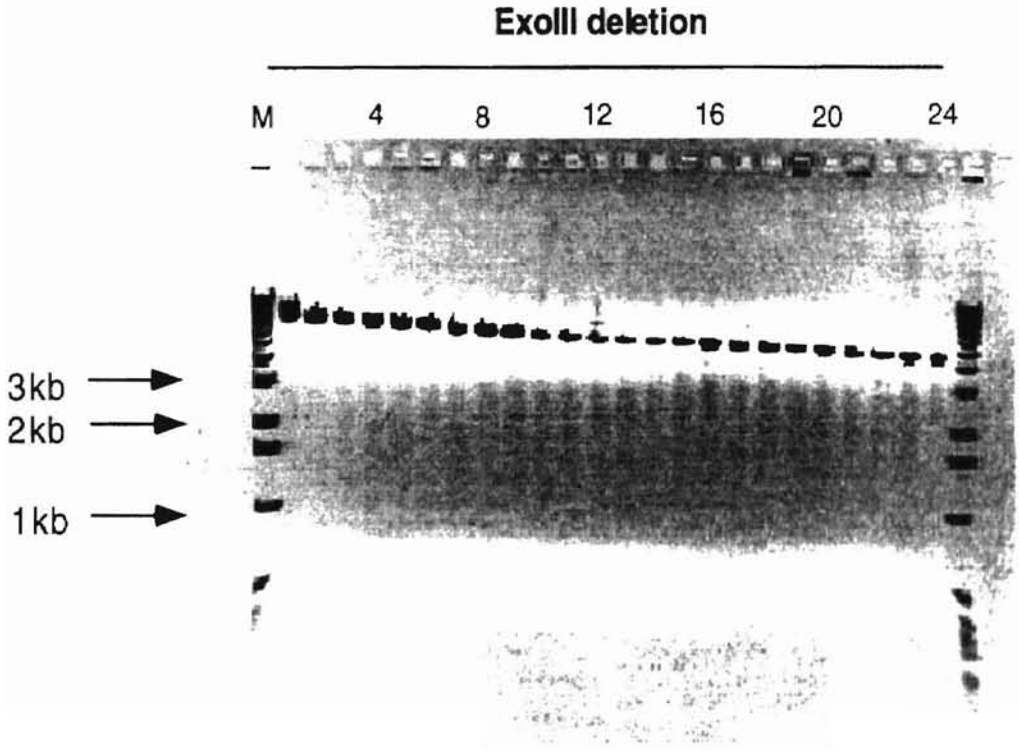

ExoIII deletion
M
4
8
12
16
20
24
3kb
2kb
1kb

Fig.10 Restriction map of plasmid pRE167 subclones and deletions.

The top line is a restriction map of the *Brucella abortus* DNA insert of pRE167 determined from the sequence. Sites for cleavage by *Eco*RI, *Kpn*I, *Hin*dIII and *Sma*I agree with digests. Inferred ORFs are indicated. The phenotype of strain RE696 containing these deletions or subclone plasmids is shown on the left.

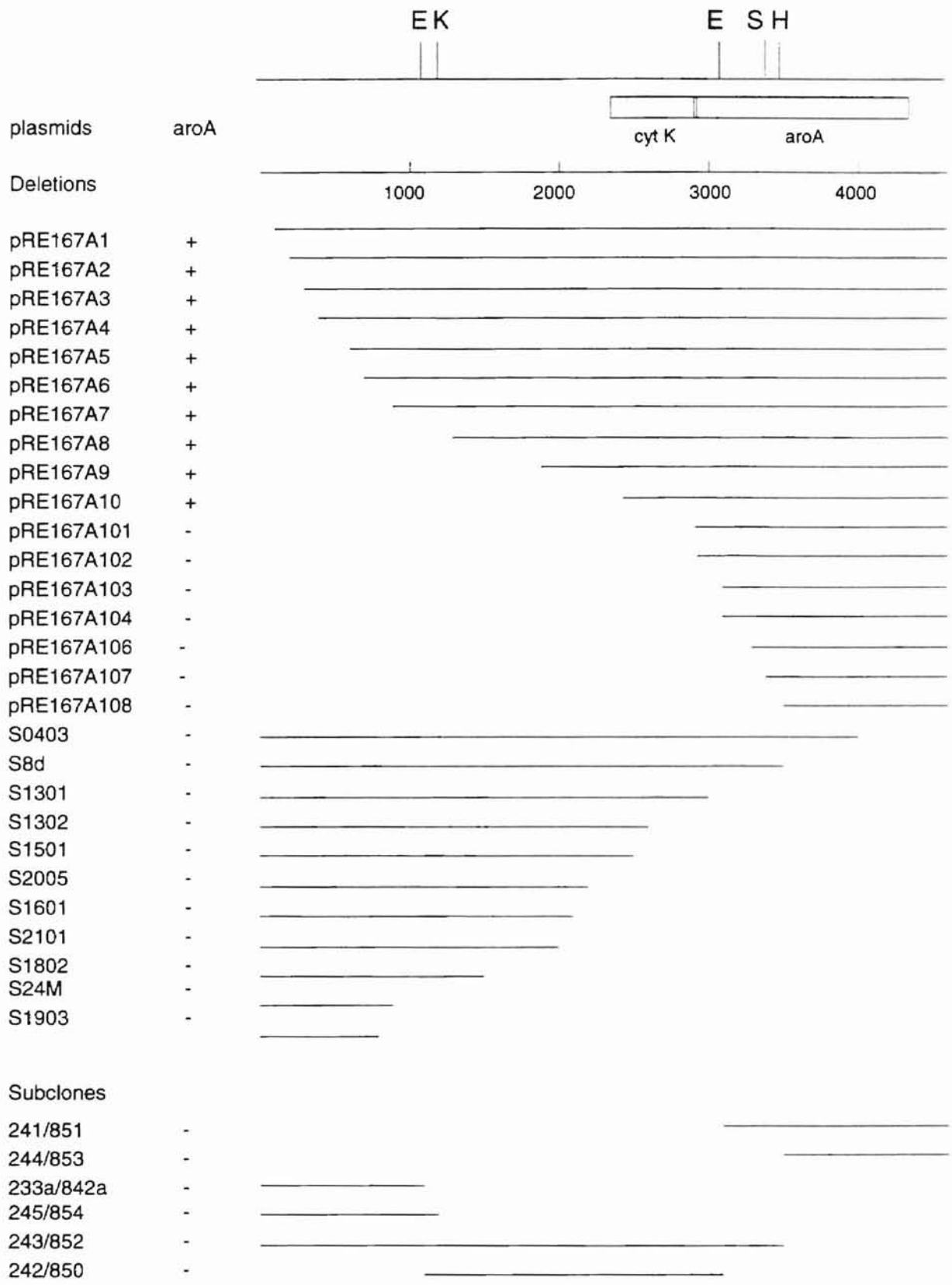

E K
E S H
cyt K
aroA
1000
2000
3000
4000
plasmids
aroA
Deletions
pRE167A1 +
pRE167A2 +
pRE167A3 +
pRE167A4 +
pRE167A5 +
pRE167A6 +
pRE167A7 +
pRE167A8 +
pRE167A9 +
pRE167A10 +
pRE167A101 -
pRE167A102 -
pRE167A103 -
pRE167A104 -
pRE167A106 -
pRE167A107 -
pRE167A108 -
S0403 -
S8d -
S1301 -
S1302 -
S1501 -
S2005 -
S1601 -
S2101 -
S1802 -
S24M -
S1903 -
Subclones
241/851 -
244/853 -
233a/842a -
245/854 -
243/852 -
242/850 -

Fig. 11 Nucleotide sequence and deduced amino acid sequence of the 2293 bp insert in pRE167A10 encoding the Brucella *aroA* protein.

The reading frame encoding the *aroA* protein begins at nucleotide 285. The cytidylate kinase reading frame starts at nucleotide 1634 just next to the stop codon of *aroA*. Amino acid residues are designated by using the single-letter code. Restriction enzyme sites *Xho*I, *Eco*RI and *Sma*I are underlined. Regions of dyad symmetry are shown as arrows below the sequence.

Sequence Range: 1 to 2290

```
        10         20         30         40         50         60         70         80         90        100
         *          *          *          *          *          *          *          *          *          *
CTCGATCTGCCCGGTATCGTCATCCCACTGTGCAGGCAATTGAAATGCCG GACAAGTGGTTAAAGCGGGCGTAGGGCGCGCTTTTACAGTGTTGTCCGGT

       110        120        130        140        150        160        170        180        190        200
         *          *          *          *          *          *          *          *          *          *
CCATAAACGCAATTTGGGGCGTTTGTCAAAGCGAAACACGCCAGTATATG CAGACTCTTCGCACAGCGTGTCGTCTGCGCCGCTTATTCACGGATGACCA
                                                                                                  M  T>

       210        220        230        240        250        260        270        280        290        300
         *          *          *          *          *          *          *          *          *          *
CACAATATTACTATGCTAGGGAGACTGCGCTTATCTCTCAATCCCGGGGC GTGTCCGCGCCAAAATGTGACTGTGAAAAATCCATGTCCCATTCCGCATG
T  Q  Y  Y  Y  A  R  E  T  A  L  I  S  Q  S  R  G   V  S  A  P  K  C  D  C  E  K  S  M  S  H  S  A  C>

       310        320        330        340        350        360        370        380        390        400
         *          *          *          *          *          *          *          *          *          *
CCCGAAACCAGCAACCGCCCGCCATTCGCAGGCACTTACGGGCGAAATCC GCATTCCGGGCGATAAATCCATTTCCACCCGCTCCTTCATGTTTGGTGGC
   P  K  P  A  T  A  R  H  S  Q  A  L  T  G  E  I   R  I  P  G  D  K  S  I  S  T  R  S  F  M  F  G  G>

       410        420        430        440        450        460        470        480        490        500
         *          *          *          *          *          *          *          *          *          *
CTTGCATCGGGTAAAACCCGCATCACCGGCCTGCTTGAGGGCGAAGACGT TATCAACACCGGGCGTGCCATGCAGGCCATGGGTGCCAGGATTCGCAAGG
   L  A  S  G  K  T  R  I  T  G  L  L  E  G  E  D  V   I  N  T  G  R  A  M  Q  A  M  G  A  R  I  R  K>

       510        520        530        540        550        560        570        580        590        600
         *          *          *          *          *          *          *          *          *          *
AAGGCGATGTCTGGATCATCAATGGTGTCGGCAATGGCTGCCTGTTGCAG CCCGAAGCGCCGCTTGATTTCGGCAATGCCGGAACCGGCGCGCGCCTGAC
E  G  D  V  W  I  I  N  G  V  G  N  G  C  L  L  Q   P  E  A  P  L  D  F  G  N  A  G  T  G  A  R  L  T>

       610        620        630        640        650        660        670        680        690        700
         *          *          *          *          *          *          *          *          *          *
CATGGGCCTCGTCGGCACGTATGATATGAAGACGTCCTTTATCGGCGATG CCTCGCTTTCCAAGCGCCCGATGGGCCGCGTGCTGAACCCGCTGCGTGAA
   M  G  L  V  G  T  Y  D  M  K  T  S  F  I  G  D   A  S  L  S  K  R  P  M  G  R  V  L  N  P  L  R  E>

       710        720        730        740        750        760        770        780        790        800
         *          *          *          *          *          *          *          *          *          *
ATGGGTGTTCAGGTTGAGGCCGCCGAAGGCGACCGGATGCCGCTGACGCT GATTGGCCCAAGGACGGCCAACCCGATCGCCTATCGCGTGCCGATGGCTT
   M  G  V  Q  V  E  A  A  E  G  D  R  M  P  L  T  L   I  G  P  R  T  A  N  P  I  A  Y  R  V  P  M  A>
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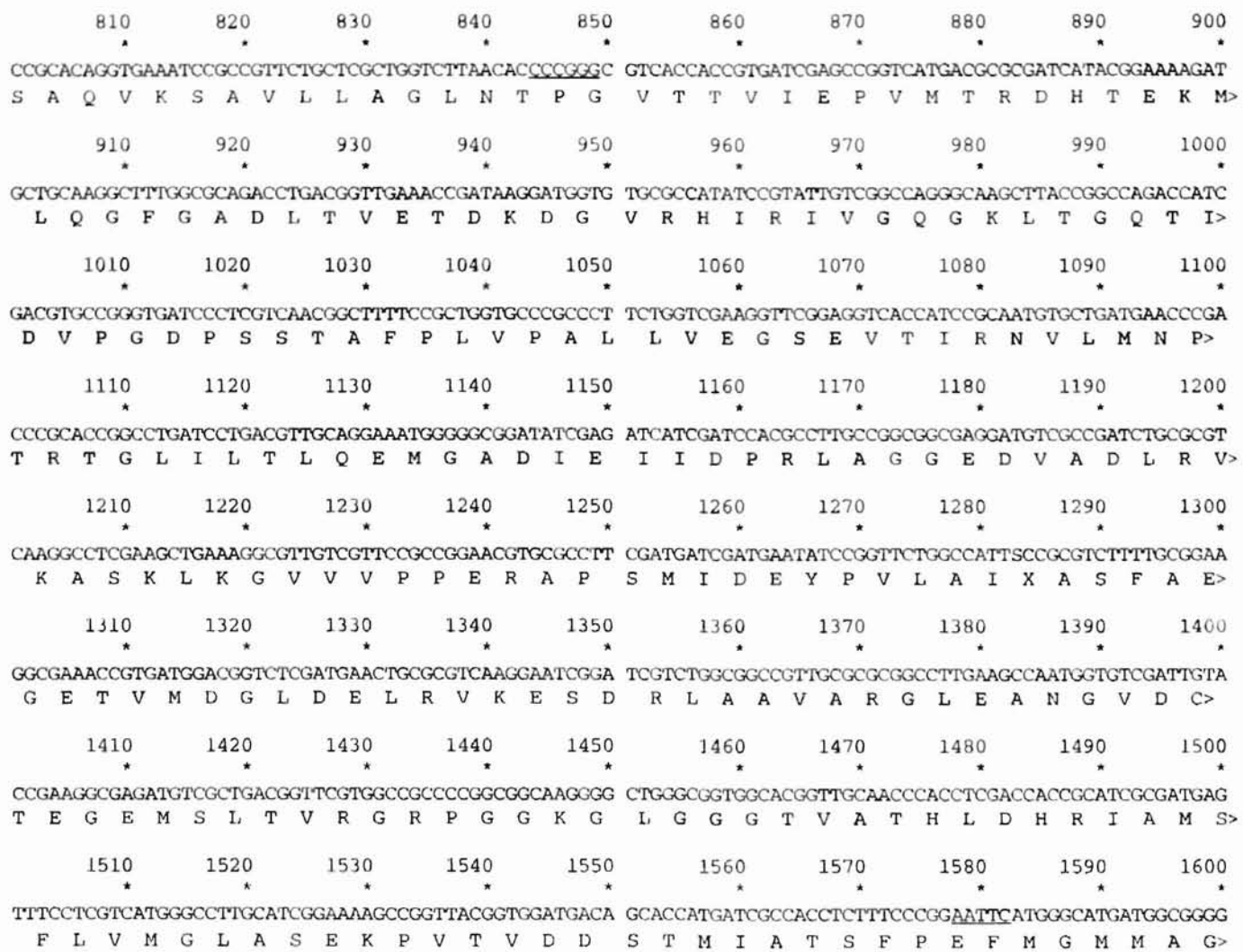
810 820 830 840 850 860 870 880 890 900
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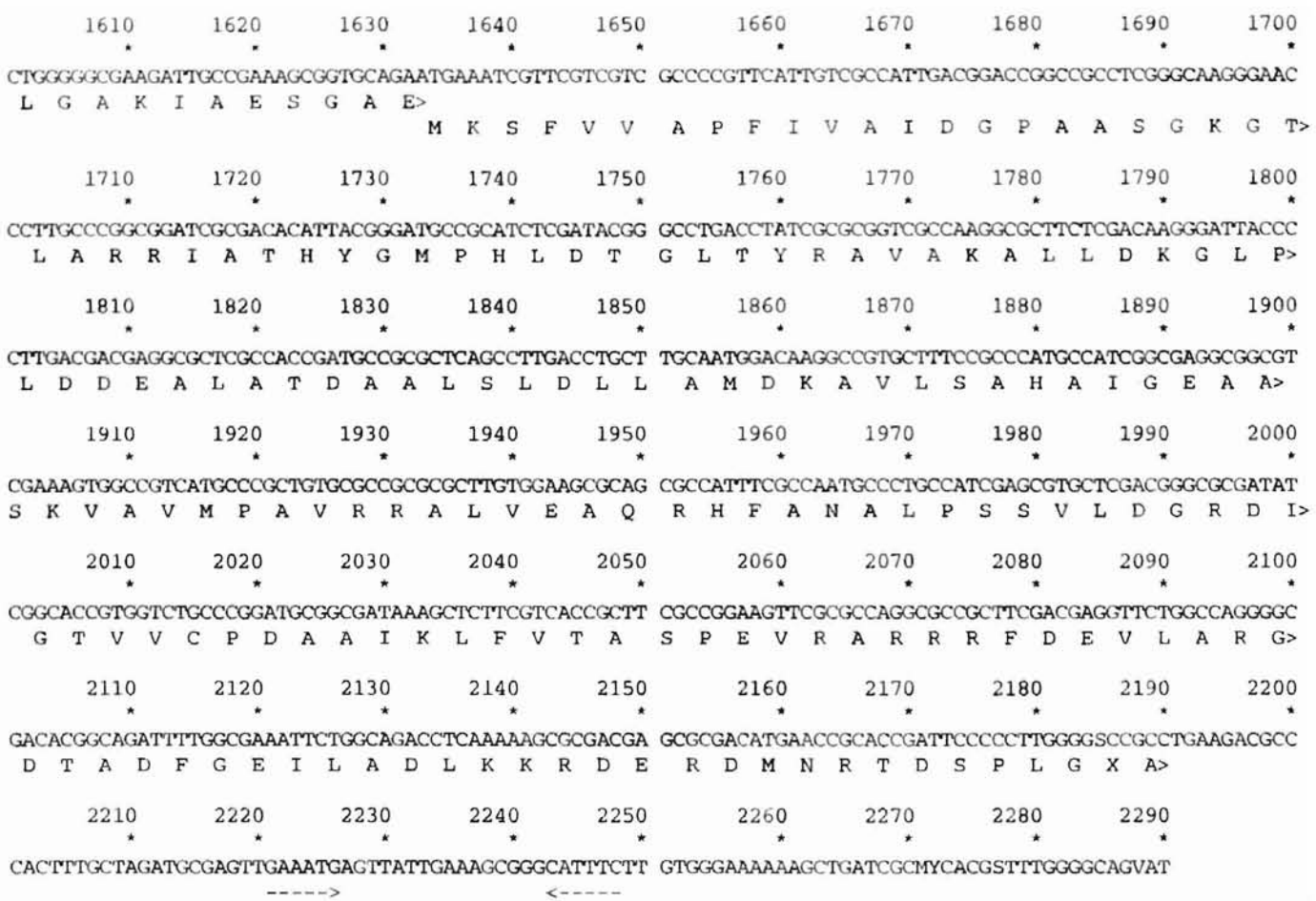
S A Q V K S A V L L A G L N T P G V T T V I E P V M T R D H T E K M>
910 920 930 940 950 960 970 980 990 1000
GCTGCAAGGCTTTGGCGCAGACCTGACGGTTGAAACCGATAAGGATGGTG TGCGCCATATCCGTATTGTCGGCCAGGGCAAGCTTACCGGCCAGACCATC
L Q G F G A D L T V E T D K D G V R H I R I V G Q G K L T G Q T I>
1010 1020 1030 1040 1050 1060 1070 1080 1090 1100
GACGTGCCGGGTGATCCCTCGTCAACGGCTTTTCCGCTGGTGCCCGCCCT TCTGGTCGAAGGTTCGGAGGTCACCATCCGCAATGTGCTGATGAACCCGA
D V P G D P S S T A F P L V P A L L V E G S E V T I R N V L M N P>
1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
CCCGCACCGGCCTGATCCTGACGTTGCAGGAAATGGGGGCGGATATCGAG ATCATCGATCCACGCCTTGCCGGCGGCGAGGATGTCGCCGATCTGCGCGT
T R T G L I L T L Q E M G A D I E I I D P R L A G G E D V A D L R V>
1210 1220 1230 1240 1250 1260 1270 1280 1290 1300
CAAGGCCTCGAAGCTGAAAGGCGTTGTCGTTCCGCCGGAACGTGCGCCTT CGATGATCGATGAATATCCGGTTCTGGCCATTSCCGCGTCTTTTGCGGAA
K A S K L K G V V V P P E R A P S M I D E Y P V L A I X A S F A E>
1310 1320 1330 1340 1350 1360 1370 1380 1390 1400
GGCGAAACCGTGATGGACGGTCTCGATGAACTGCGCGTCAAGGAATCGGA TCGTCTGGCGGCCGTTGCGCGCGGCCTTGAAGCCAATGGTGTCGATTGTA
G E T V M D G L D E L R V K E S D R L A A V A R G L E A N G V D C>
1410 1420 1430 1440 1450 1460 1470 1480 1490 1500
CCGAAGGCGAGATGTCGCTGACGGTTCGTGGCCGCCCCGGCGGCAAGGGG CTGGGCGGTGGCACGGTTGCAACCCACCTCGACCACCGCATCGCGATGAG
T E G E M S L T V R G R P G G K G L G G G T V A T H L D H R I A M S>
1510 1520 1530 1540 1550 1560 1570 1580 1590 1600
TTTCCTCGTCATGGGCCTTGCATCGGAAAAGCCGGTTACGGTGGATGACA GCACCATGATCGCCACCTCTTTCCCGGAATTCATGGGCATGATGGCGGGG
F L V M G L A S E K P V T V D D S T M I A T S F P E F M G M M A G>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L G A K I A E S G A E>
M K S F V V A P F I V A I D G P A A S G K G T>
1710 1720 1730 1740 1750 1760 1770 1780 1790 1800
CCTTGCCCGGCGGATCGCGACACATTACGGGATGCCGCATCTCGATACGG GCCTGACCTATCGCGCGGTCGCCAAGGCGCTTCTCGACAAGGGATTACCC
L A R R I A T H Y G M P H L D T G L T Y R A V A K A L L D K G L P>
1810 1820 1830 1840 1850 1860 1870 1880 1890 1900
CTTGACGACGAGGCGCTCGCCACCGATGCCGCGCTCAGCCTTGACCTGCT TGCAATGGACAAGGCCGTGCTTTCCGCCCATGCCATCGGCGAGGCGGCGT
L D D E A L A T D A A L S L D L L A M D K A V L S A H A I G E A A>
1910 1920 1930 1940 1950 1960 1970 1980 1990 2000
CGAAAGTGGCCGTCATGCCCGCTGTGCGCCGCGCGCTTGTGGAAGCGCAG CGCCATTTCGCCAATGCCCTGCCATCGAGCGTGCTCGACGGGCGCGATAT
S K V A V M P A V R R A L V E A Q R H F A N A L P S S V L D G R D I>
2010 2020 2030 2040 2050 2060 2070 2080 2090 2100
CGGCACCGTGGTCTGCCCGGATGCGGCGATAAAGCTCTTCGTCACCGCTT CGCCGGAAGTTCGCGCCAGGCGCCGCTTCGACGAGGTTCTGGCCAGGGGC
G T V V C P D A A I K L F V T A S P E V R A R R R F D E V L A R G>
2110 2120 2130 2140 2150 2160 2170 2180 2190 2200
GACACGGCAGATTTTGGCGAAATTCTGGCAGACCTCAAAAAGCGCGACGA GCGCGACATGAACCGCACCGATTCCCCCTTGGGGSCCGCCTGAAGACGCC
D T A D F G E I L A D L K K R D E R D M N R T D S P L G X A>
2210 2220 2230 2240 2250 2260 2270 2280 2290
CACTTTGCTAGATGCGAGTTGAAATGAGTTATTGAAAGCGGGCATTTCTT GTGGGAAAAAAGCTGATCGCMYCACGSTTTGGGGCAGVAT
-----> <-----

Fig. 12 Comparison of the deduced amino acid sequence of the Brucella *aroA* protein with the published sequences of 16 other species.

The single-letter code is used for designating amino acids. Asterisks below the aligned amino acids indicate identity between the corresponding amino acid residues, dots indicate conserved amino acid replacements, and dashes between the residues represent gaps inserted by the alignment program to maximize identity between the sequences. The abbreviated bacteria names and their classifications are:

Lacla: *Lactococcus lactis*, firmicutes, gram-positive;
Synec: *Synechocystis* PCC6803; cyanobacteria;
Bruab: *Brucella abortus*; proteobacteria, alpha subdivision;
Dicno: *Dichelobacter nodosus*, proteobacteria, gamma subdivision;
Staau: *Staphylococcus aureus*, firmicutes, gram-positive;
Camje: *Campylobacter jejuni*, proteobacteria, epsilon subdivision;
Ecoli: *Escherichia coli*, proteobacteria, gamma subdivision;
Salgl: *Salmonella gallinarum*, proteobacteria, gamma subdivision;
Klepn: *Klebsiella pneumoniae*, proteobacteria, gamma subdivision;
Yeren: *Yersinia enterocolitica*, proteobacteria, gamma subdivision;
Pasha: *Pasteurella haemolyticus*, proteobacteria, gamma subdivision;
Haein: *Haemophilus influenza*, proteobacteria, gamma subdivision;
Pasmu: *Pasteurella multocida*, proteobacteria, gamma subdivision;
Aersa: *Aeromonas salmonicida*, proteobacteria, gamma subdivision;
Borpe: *Bordetella pertussis*, proteobacteria, beta subdivision;
Psepm: *Burkholderia pseudomollei*, proteobacteria, beta subdivision;
Myctu: *Mycobacterium tubercucolosis*, firmicutes, gram-positive.

```
Lacla      ----------------------MK------------------------LKINSQ-----G
Synec      ----------------------MALLSLNNHQSHQR------------LTVNPPAQG-VA
Bruab      ------------MTTQYYYARETALISQSRGVSAPKCDCEKSMSHSACPKPATARHS-QA
Dicno      ------------------------------------------------MMTNIWHTAPV-SA
Staau      ------------------------------------------------MVNEQIIDIS-GP
Camje      ------------------------------------------------MKIYKLQT---P
Ecoli      ------------------------------------------------MESLTLQPI-AR
Salgi      ------------------------------------------------MESLTLQPI-AR
Klepn      ------------------------------------------------MESLTLQPI-AR
Yeren      ------------------------------------------------MLESLTLHPI-AL
Pasha      ------------------------------------------------MEKLTLTPI-SR
Haein      ------------------------------------------------MEKITLAPI-SA
Pasmu      ----------------------------------------------MIKDATAITLNPI-SY
Aersa      ------------------------------------------------NSLRLEPI-SR
Borpe      ----------------------------------------------MSGLAYLDLPAA-RL
Psepm      ----------------------------------------------MAFQWPRFPLQPW-RH
Myctu      ------------------------------------------------MKTWPAPTAPTP

Lacla      LKGRLK-VPGDKSISHRSIMFGSIA----KGKTIIHDILRGEDVLSTIEAFRALGVEIED
Synec      LTGRLR-VPGDKSISHRALMLGAIA----TGETIIEGLLLGEDPRSTAHCFRAMGAEISE
Bruab      LTGEIR-IPGDKSISTRSFMFGGLA----SGKTRITGLLEGEDVINTGRAMQAMGARIRK
Dicno      LSGEIT-ICGDKSMSHRALLLAALA----EGQTEIRGFLACADCLATRQALRALGVDIQR
Staau      LKGEIE-VPGDKSMTHRAIMLASLA----EGVSTIYKPLLGEDCRRTMDIFRHLGVEIKE
Camje      VNAILENIAADKSISHRFAIFSLLT----QEENKAQNYLLAQDTLNTLEIIKNLGAKIE-
Ecoli      VDGTIN-LPGSKTVSNRALLLAALA----HGKTVLTNLLDSDDVRHMLNALTALGVSYT-
Salgl      VDGAIN-LPGSKSVSNRALLLAALA----CGKTVLTNLLDSDDVRHMLNALSALGINYT-
Klepn      VDGTVN-LPGSKSVSNRALLLAALA----RGTTVLTNLLDSDDVRHMLNALSALGVHYV-
Yeren      INGTVN-LPGSKSVSNRALLLAALA----EGTTQLNNLLDSDDIRHMLNALQALGVKYR-
Pasha      VEGEIN-LPGSKSLSNRALLLAALA----TGTTQVTNLLDSDDIRHMLNALKALGVKYE-
Haein      VEGTIN-LPGSKSLSNRALLLAALA----KGTTKVTNLLDSDDIRHMLNALKALGVRYQ-
Pasmu      IEGEVR-LPGSKSLSNRALLLSALA----KGKTTLTNLLDSDDVRHMLNALKELGVTYQ-
Aersa      VAGEVN-LPGSKSVSNRALLLAALA----RGTTRLTNLLDSDDIRHMLAALTQLGVKYK-
Borpe      ARGEVA-LPGSKSISNRVLLLAALA----EGSTEITGLLDSDDTRVMLAALRQLGVSVG-
Psepm      VTGHLR-LPGDKSISNRSLLLGALA----EGVTEVTGLLDSDDARAMLNALRDLGVVIE-
Myctu      VRATVT-VPGSKSQTNRALVLAALAAAQGRGASTISGALRSRDTELMLDALQTLGLRVDG

Lacla      -DGQVIT----VHGQGISKLKEPEKALDMGN------SGTSTRLLSGILA-------GL-
Synec      LNSEKII----VQGRGLGQLQEPSTVLDAGN------SGTTMRLMLGLLA-------GQK
Bruab      -EGDVWI----INGVGNGCLLQPEAPLDFGN------AGTGARLTMGLV--------GTY
Dicno      -EKEIVT----IRGVGFLGLQPPKAPLNMQN------SGTSMRLLAGILA-------AQ-
Staau      -DDEKLV----VTSPGY-QVNTPHQVLYTGN------SGTTTRLLAGLLS-------GLG
Camje      -QKDSCVKI--IPPKE---ILSPNCILDCGN------SGTAMRLMIGFLA-------GIS
Ecoli      LSADRTRCE--IIGNGGPLHAEGALELFLGN------AGTAMRPLAAALCL------GS-
Salgl      LSADRTRCD--ITGNGGPLRAPGALELFLGN------AGTAMRPLAAALCL------GQ-
Klepn      LSSDRTRCE--VTGTGGPLQAGSALELFLGN------AGTAMRPLAAALCL------GS-
Yeren      LSADRTRCE--VDGLGGKLVAEQPLELFLGN------AGTAMRPLAAALCL------GK-
Pasha      LSDDKTVCV--LEGIGGAFKVQNGLSLFLGN------AGTAMRPLAAALCLKG---EEK-
Haein      LSDDKTICE--IEGLGGAFNIQDNLSLFLGN------AGTAMRPLTAALCLKG---NHE-
Pasmu      LSEDKSVCE--IEGLGRAFEWQSGLALFLGN------AGTAMRPLTAALCLSTPNREGK-
Aersa      LSADKTECT--VHGLGRSFAVSAPVNLFLGN------AGTAMRPLCAALCL------GS-
Borpe      EVADGC-----VTIEGVARFPTEQAELFLGN------AGTAFRPLTAALALMG-------
Psepm      -GPHQGRCT--VHGVGLHGLKAPPGPLFLGN------AGTAMRPLSAALALQPF------
Myctu      VGSELT-----VSGRIE--P-GPGARVDCGL------AGTVLRFVPPLAAL--------G
```

```
Lacla     PFEATLFGDDSLSKRPMDRVATPLQMMGAEIVGQTDKVKLPMTIKGSAHLKAIDYILPVA
Synec     DCLFTVTGDDSLRHRPMSRVIQPLQQMGAKIWARSNGKFAPLAVQGS-QLKPIHYHSPIA
Bruab     DMKTSFIGDASLSKRPMGRVLNPLREMGVQVEA-AEGDRMPLTLIGPRTANPIAYRVPMA
Dicno     RFESVLCGDESLEKRPMQRIITPLVQMGAKIVSHSNFT-APLHISG-RPLTGIDYALPLP
Staau     N-ESVLSGDVSIGKRPMDRVLRPLKLMDANIEG-IEDNYTPLIIKPS-VIKGINYQMEVA
Camje     GF-FVLSGDKYLNNRPMRRISKPLTQIGARIYGRNEANLAPLCIEG-QNLKAFNYKSEIS
Ecoli     -NDIVLTGEPRMKERPIGHLVDALRLGGAKITYLEQENYPPLRLQG-GFT-GGNVDVDGS
Salgl     -NEIVLTGEPRMKERPIGHLVDSLRQGGANIDYLEQENYPPLRLRG-GFI-GGDIEVDGS
Klepn     -NDIVLTGEPRMKERPIGHLVDALRQGGAQIDYLEQENYPPLRLRG-GFT-GGDVEVDGS
Yeren     -NDIVLTGEPRMKERPIGHLVDALRQGGAQIDYLEQENYRR-CIAG-GFR-GGKLTVDGS
Pasha     -SQIILTGEPRMKERPIKHLVDALRQVGAEVQYLENEGYPPLAISNSVCR-GGKVQIDGS
Haein     -VEIILTGEPRMKERPILHLVDALRQAGADIRYLENEGYPPLAIRNKGIK-GGKVKIDGS
Pasmu     -NEIVLTGEPRMKERPIQHLVDALCQAGAEIQYLEQEGYPPIAIRNTGLK-GGRIQIDGS
Aersa     -GEYMLGGEPRMEERPIGHLVDCLALKGAHIQYLKKDGYPPLVVDAKGLW-GGDVHVDGS
Borpe     -GDYRLSGVPRMHERPIGDLVDALRQFGAGIEYLGQAGYPPLRIGGGSIRVDGPVRVEGS
Psepm     --DTTLTGDPRMSERPINRLVDALREMGAVIEYLAQEGYPPLTIRG----------GGS
Myctu     SVPVTFDGDQQARGRPIAPLLDALRELGVAVD----GTGLPFRVRGNGSLAGGTVAIDAS
                 *     **.  .   *      .            .

Lacla     -SAQVKSAVIFAA------LQAEGLTKVVEKEKTRSHTEEMLVQFGGEITVS---DKT--
Synec     -SAQVKSCLLLAG------LTTEGDTTVTEPALSRDHSERMLQAFGAKLTID---PVTHS
Bruab     -SAQVKSAVLLAG------LNTPGVTTVIEPVMTRDHTEKMLQGFGADLTVETDKDGVRH
Dicno     -SAQLKSCLILAG------LLADGTTRLHTCGISRDHTERMLPLFGGALEIKKE-----Q
Staau     -SAQVKSAILFAS------LFSKEPTIIKELDVSRNHTETMFKHFN----IPIEAEGLSI
Camje     -SAQVKTAMILSA------FRANNVCAFSEISLSRNHSENMLKAMKAPIRVS--NDGLSL
Ecoli     VSSQFLTALLMTAP-LA---PEDTVIRIKGDLVSKPYIDITLNLMK-TFGVEIENQHYQQ
Salgl     VSSQFLTALLMTAP-LA---PKDTIIRVKGELVSKPYIDITLNLMK-TFGVEIANHHYQQ
Klepn     VSSQFLTALLMASP-LA---PQDTVLAIKGELVSRPYIDITLHLMK-TFGVEVENQAYQR
Yeren     VSSQFLTALLMTAP-LA---EQDTEIQIQGELVSKPYIDITLHLMK-AFGVDVVHENYQI
Pasha     ISSQFLTALLMSAP-LA---EGDMEIEIIGDLVSKPYIDITLSMMN-DFGITVENRDYKT
Haein     ISSQFLTALLMSAP-LA---ENDTEIEIIGELVSKPYIDITLAMMR-DFGVKVENHHYQK
Pasmu     VSSQFLTALLMAAP-MA---EADTEIEIIGELVSKPYIDITLKMMQ-TFGVEVENQAYQR
Aersa     VSSQFLTAFLMAAPAMA---PVIPRIHIKGELVSKPYIDITLHIMN-SSGVVIEHDNYKL
Borpe     VSSQFLTALLMAAPVLARRSGQDITIEVVGELISKPYIEITLNLMA-RFGVSVRRDGWRA
Psepm     VSSQFLTALLMTAPMASAQIKS-------GLLLSKPYIDITLNVM--PFGVPTRDHTERI
Myctu     ASSQFVSGLLLSAASFT---DGLTVQHTGSSLPSAPHIAMTAAMLR-QAGVDIDDSTPNR
           *.*  .  . .                    .                .

Lacla     ILVPGGQK---LLGQ---EVTVPGDISSAAFWLVAGLVVENSGLILENVGINETRTG--I
Synec     VTVHGPAH---LTGQ---RVVVPGDISSAAFWLVAASILPGSELLVENVGINPTRTG--V
Bruab     IRIVGQGK---LTGQ---TIDVPGDPSSTAFPLVPALLVEGSEVTIRNVLMNPTRTG--L
Dicno     IIVTGGQK---LHGC---VLDIVGDLSAAAFFMVAALIAPRAEVVIRNVGINPTRAA--I
Staau     NTTPEAIR---YIKPA--DFHVPGDISSAAFFIVAALITPGSDVTIHNVGINQTRSG--I
Camje     EISPLKKP---LKAQ---NIIIPNDPSSAFYFALAAIILPKSQIILKNILLNPTRIE--A
Ecoli     FVVKGGQS---YQSP--GTYLVEGDASSASYFLAAAAI-KGGTVKVTGIGRNSMQGDIRF
Salgl     FVVKGGQQ---YHSP--GRYLVEGDASSASYFLAAGAI-KGGTVKVTGIGRKSMQGDIRF
Klepn     FIVRGNQQ---YQSP--GDYLVEGDASSASYFLAAGAI-KGGTVKVTGIGRNSVQGDIRF
Yeren     FHIKGGQT---YRSP--GIYLVEGDASSASYFLAAAAI-KGGTVRVTGIGKQSVQGDTKF
Pasha     FLVKGKQG---YVAPQ-GNYLVEGDASSASYFLASGAI-K-A-GKVTGIGKKSIQGDRLF
Haein     FQVKGNQS---YISP--NKYLVEGDASSASYFLAAGAI-K-GKVKVTGIGKNSIQGDRLF
Pasmu     FLVKGHQQ---YQSPH--RFLVEGDASSASYFLAAAAI-K-GKVKVTGVGKNSIQGDRLF
Aersa     FYIKGNQS---IVSP--GDFLVEGDASSASYFLAAGAI-K-GKVRVTGIGKHSI-GDIHF
Borpe     FTIARDAV---YRGP--GRMAIEGDASTASYFLALGAI-GGGPVRVTGVGEDSIQGDVAF
Psepm     FAVSAIR----YPSP--AVLRLEGDATSASYFLAAAGI-KG--VPVTGIGRHSMQGDSWF
Myctu     WQVRPGPV-------AARRWDIEPDLTNAVAFLSAAVV-SGGTVRITGWPRVSVQPADHI
                                 .  *  .  .        .        .  .
```

```
Lacla     LEVIQAMGGQLEILEQDEVA--KAATLKVK-ASQLK------GTEISGDLIPRLIDELPI
Synec     LEVLAQMGADITPENERLVTGEPVADLRVR-ASHLQ------GCTFGGEIIPRLIDEIPI
Bruab     ILTLQEMGADIEIIDPRLAGGEDVADLRVK-ASKLK------GVVVPPERAPSMIDEYPV
Dicno     ITLLQKMGGRIELHHQRFWGAEPVADIVVY-HSKLR------GITVAPEWIANAIDELPI
Staau     IDIVEKMGGNIQLFNQT-TGAEPTASIRIQYTPMLQ------PITIEGELVPKAIDELPV
Camje     YKILQKMGAKLEMTITQ-NDFETIGEIRVE-SSKLN------GIEVKDN-IAWLIDEAPA
Ecoli     ADVLEKMGATICWGDDYI---S------CTRGELN-------AIDMDMNHIPDAAMTIAT
Salgl     ADVLEKMGATITWGDDFI---A------CTRGELH-------AIDMDMNHIPDAAMTIAT
Klepn     ADVLEKMGATVTWGEDYI---A------CTRGELN-------AIDMDMNHIPDAAMTIAT
Yeren     ADVLEKMGAKISWGDDYI---E------CSRGELQ-------GIDMDMNHIPDAAMTIAT
Pasha     ADVLEKMGAKITWGEDFI---Q------AEQSPLK-------GVDMDMNHIPDAAMTIAT
Haein     ADVLEKMGAKITWGEDFI---Q------AEHAELN-------GIDMDMNHIPDAAMTIAT
Pasmu     ADVLEKMGAHITWGDDFI---Q------VEKGNLK-------GIDMDMNHIPDAAMTIAT
Aersa     ADVLERMGARITWGDDFI---E------AEQGPLH-------GVDMDMNHIPDVGHDHSG
Borpe     AATLAAMGADVRYGPGWI---ETRGVRVAEGGRLK-------AFDADFNLIPDAAMTAAT
Psepm     PRALRRMGARSCGSSMIV----------CPRGELRA------AVRSDSNSIPDAAMTLAT
Myctu     LAILRQLNAVVIHADSSL---------EVRGPTGYD------GFDVDLRAVGELTPSVAA
                . . ..

Lacla     IALLATQAEGKTIIRDAAELKVKETDRIAVVADALNSMGANIEPTDDG------MIIQGG
Synec     LAVAAAFAEGTTRIEDAAELRVKESDRLAAIASELGKMGAKVTEFDDG------LEIQGG
Bruab     LAIXASFAEGETVMDGLDELRVKESDRLAAVARGLEANGVDCTEGEMS------LTVRGR
Dicno     FFIAAACAEGTTFVGNLSELRVKESDRLAAMAQNLQTLGVACDVGADF------IHIYGR
Staau     IALLCTQAVGTSTIKDAEELKVKETNRIDTTADMLNLLGFELQPTNDG------LIIHPS
Camje     LAIAFALAKGKSSLINAKELRVKESDRIAVMVENLKLCGVEARELDDG------FEIEGG
Ecoli     AALFAK-GTTR--LRNIYNWRVKETDRLFAMATELRKVGAEV---EEGHD----YIRITP
Salgl     TALFAK-GTTT--LRNIYNWRVKETDRLFAMATELRKVGAEV---EEGHD----YIRITP
Klepn     AALFAR-GTTT--LRNIYNWRVKETDRLFAMATELRKVGAEV---EEGED----YIRITP
Yeren     TALFAD-GPTV--IRNIYNWRVKETDRLSAMATELRKVGAEV---EEGQD----YIRVVP
Pasha     TALFAE-GETV--IRNIYNWRVKETDRLTAMATELRKVGAEVEEGEEGED----FIRIQP
Haein     TALFSN-GETV--IRNIYNWRVKETDRLTAMATELRKVGAEV---EEGED----FIRIQP
Pasmu     TALFAE-GETV--IRNIYNWRVKETDRLTAMATELRKVGAEV---EEGED----FIRIQP
Aersa     QSHCLP-RVPP--HSQHLQLAVRD-DRCTPCTHGHRRAQAGV--SEEGTT----FITRDA
Borpe     LALYAD-GPCR--LRNIGSWRVKETDRIHAMHTELEKLGAGV---QSGAD----WLEVAP
Psepm     RSAGAR-WAAT--ANHI---RVAGEGDGSAVC-NVHGAGGGWRASGSRCW----SSWLPS
Myctu     LAALAS-PGSVSRLSGIAHLRGHETDRLAALSTEINRLGGTCRETPDG------LVITAT

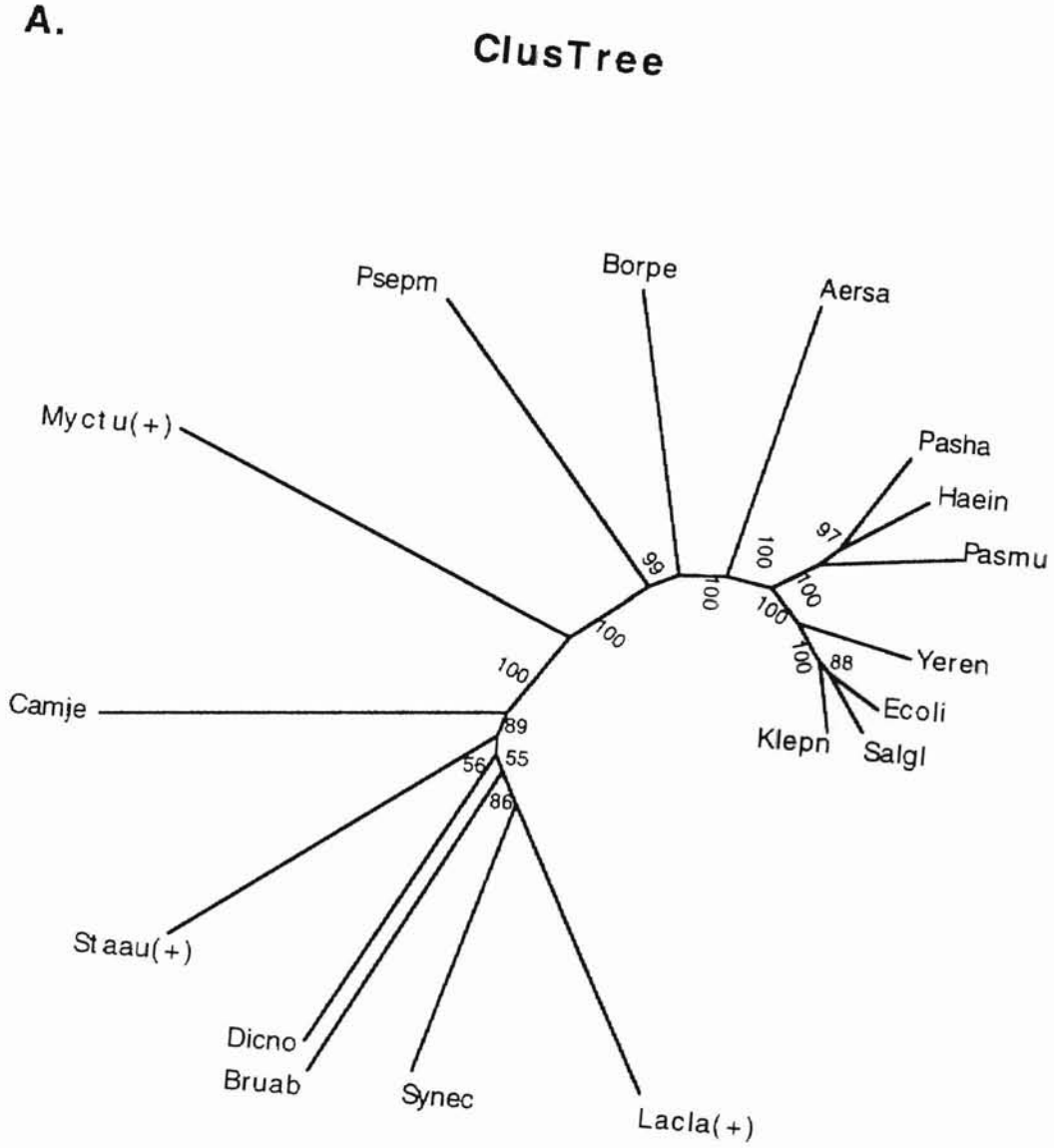
Lacla     TKLHA-PENAINTLGDHRIGMMVAIAALLVENGEIELERAEAIQTSYPSFFDDLEKLSGN
Synec     SPLQG-AE--VDSLTDHRIAMALAIAA-LGSGGQTIINRAEAAAISYPEFFGTLGQVAQG
Bruab     PGGKGLGGGTVATHLDHRIAMSFLVMG-LASEKPVTVDDSTMIATSFPEFMGMMAGLGAK
Dicno     SDRQFLPAR-VNSFGDHRIAMSLAVAG-VRAAGELLIDDGAVAAVSMPQFRDFAAAIGMN
Staau     E----FKTNATDILTDHRIGMMLAVACVLSSE-PVKIKQFDAVNVSFPGFLPKLKLLQNE
Camje     CE---LKSSKIKSYGDHRIAMSFAILGLLCG---IEIDDSDCIKTSFPNFIEILSNLGAR
Ecoli     PE--KLNFAEIATYNDHRMAMCFSLVAL--SDTPVTILDPKCTAKTFPDYFEQLARISQA
Salgl     PA--KLQHADIGTYNDHRMAMCFSLVAL--SDTPVTILDPKCTAKTFPDYFEQLARMSTP
Klepn     PL--TLQFAEIGTYNDHRMAMCFSLVAL--SDTPVTILDPKCTAKTFPDYFGQLARISTL
Yeren     PA--QLIAAEIGTYNDHRMAMCFSLVAL--SDTPVTILDPKCTAKTFPDYFEQLARLSQI
Pasha     LALENFQHAEIETYNDHRMAMCFSLIAL--SNTEVTILDPNCTAKTFPTYFRDLEKLSVR
Haein     LALNQFKHANIETYNDHRMAMCFSLIAL--SNTPVTILDPKCTAKTFPTFFNEFEKICLK
Pasmu     LNLAQFQHAELNI-HDHRMAMCFALIAL--SKTSVTILDPSCTAKTFPTFLILF-TLNTR
Aersa     ADPAQARRDR--HLQRSRIAMCFSLVAL--SDIAVTINDPGCTSKTFPDYFDKLASVSQA
Borpe     PEPGGWRDAHIGTWDDHRMAMCFLLAAF--GPAAVRILDPGCVSKTFPDYFDVYAGLLAA
Psepm     ARKVVLRCAVPKRFPDGNVLLAAS--AW--RTGCETSWIP--AAPTRRRIVIEGGAIGS-
Myctu     P----LRPGIWRAYADHRMAMAGAIIGL--RVAGVEVDDIAATTKTLPEFPRLWAEMVGP
                                  . .                .          .
```

```
Lacla      L-------------------------
Synec      -------------------------
Bruab      IAESGAE-------------------
Dicno      VGEKDAKNCHD---------------
Staau      G-------------------------
Camje      IDY-----------------------
Ecoli      A-------------------------
Salgl      A-------------------------
Klepn      A-------------------------
Yeren      A-------------------------
Pasha      -------------------------
Haein      N-------------------------
Pasmu      EVAYR---------------------
Aersa      V-------------------------
Borpe      RD------------------------
Psepm      -------------------------
Myctu      GQGWGYPQPRSGQRARRATGQGSGG-
```

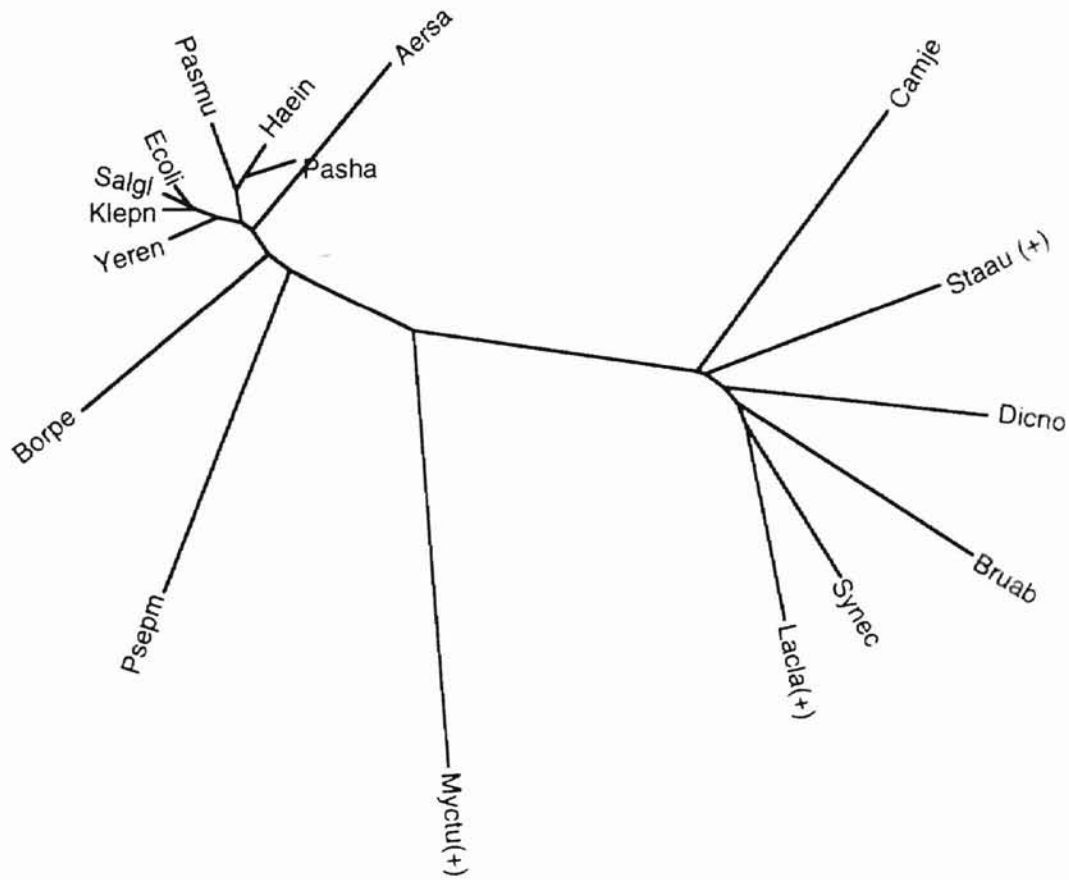
Fig. 13 Phylogenetic trees generated by different programs.

A. Clustree was generated by neighbor joining method. Bootstrap values are showed beside the corresponding internal branches. B. Fitchtree was generated by distance method. C. Parstree was generated by parsimony method. Branch lengths are proportional to distances for Fitchtree and Clustree but not for Parstree. (+) indicates gram-positive bacteria.

A.


ClusTree
Psepm
Borpe
Aersa
Myctu(+)
Pasha
Haein
Pasmu
Yeren
Ecoli
Salgl
Klepn
Camje
Staau(+)
Dicno
Bruab
Synec
Lacla(+)
99
100
100
97
100
100
100
88
100
89
56
55
86

B. **FitchTree**


Pasmu
Haein
Aersa
Ecoli
Salgl
Pasha
Klepn
Yeren
Camje
Staau (+)
Borpe
Dicno
Bruab
Psepm
Synec
Lacla(+)
Myclu(+)

C

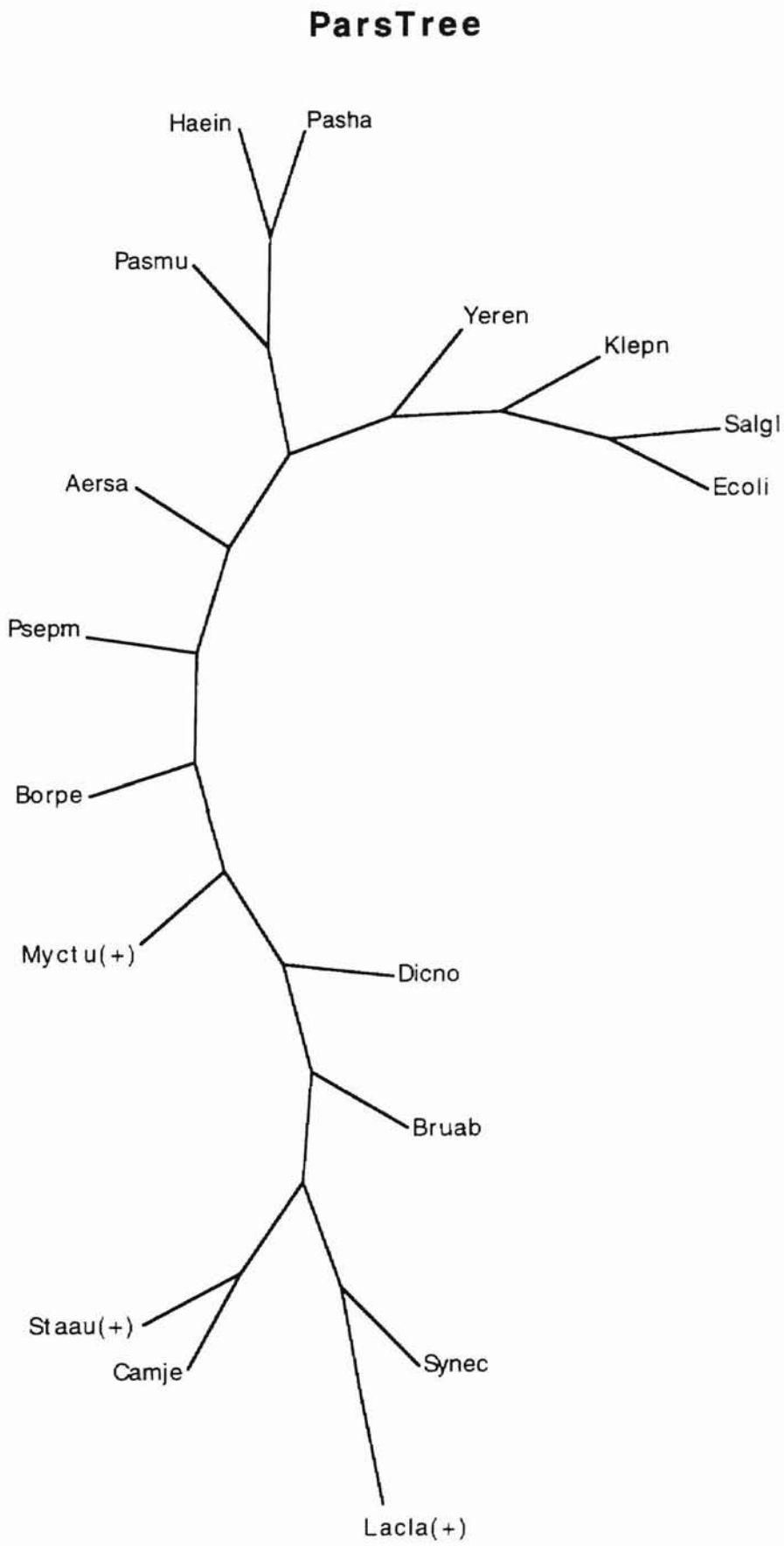

ParsTree
Haein
Pasha
Pasmu
Yeren
Klepn
Salgl
Ecoli
Aersa
Psepm
Borpe
Myctu(+)
Dicno
Bruab
Staau(+)
Camje
Synec
Lacla(+)

Fig. 14 One-dimensional SDS-PAGE of proteins labeled in maxicells containing pRE167 and various deletions.

The positions of the prestained protein standards and their apparent molecular masses are indicated on the right.

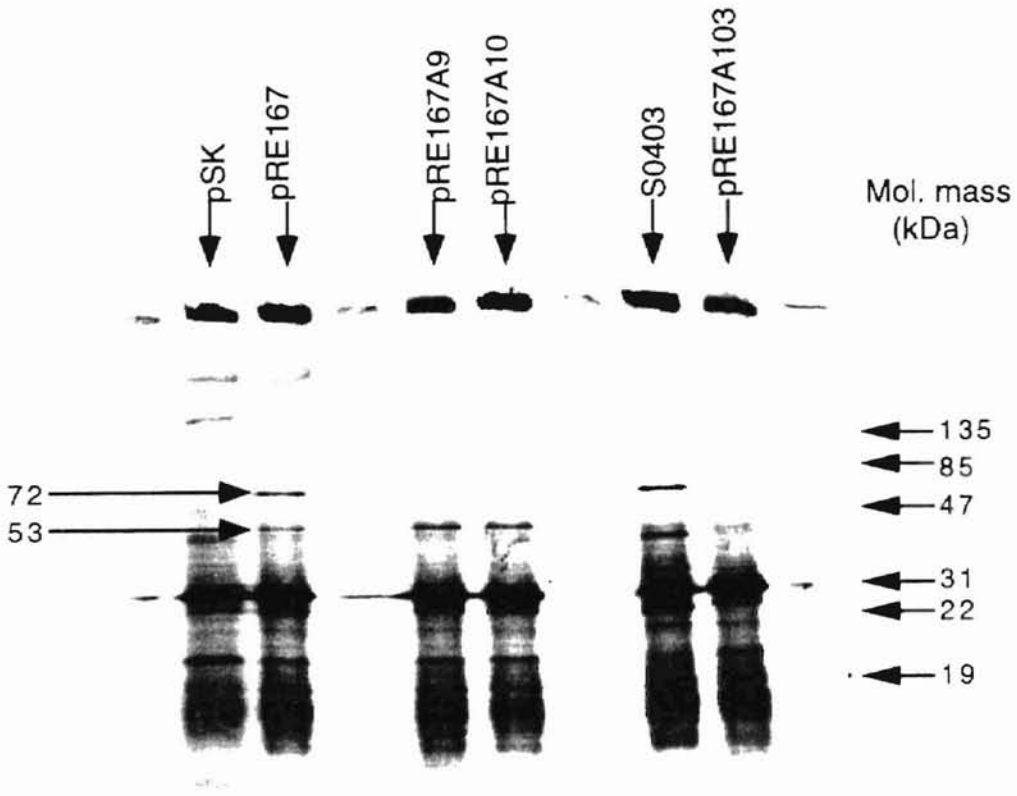
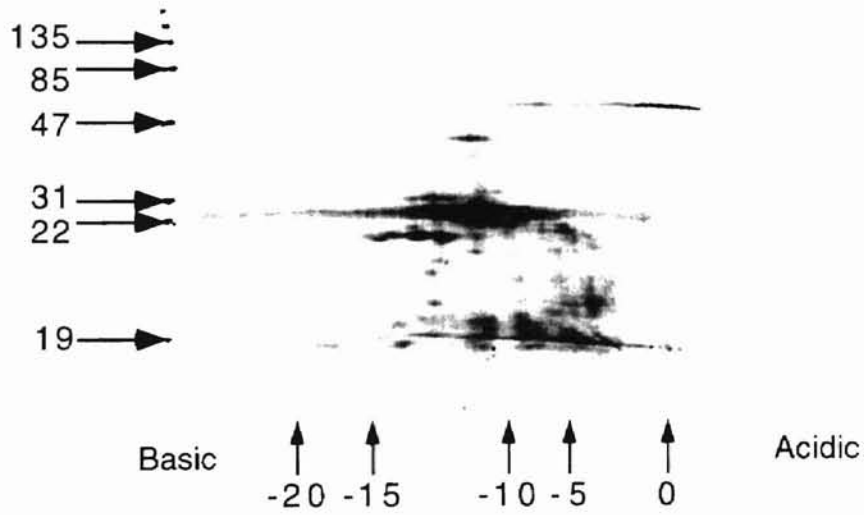

pSK
pRE167
pRE167A9
pRE167A10
S0403
pRE167A103
Mol. mass
(kDa)
72
53
135
85
47
31
22
19

Fig. 15 Two-dimensional gel electrophoresis of proteins labeled in maxicells containing pRE167 and pRE167A103.

The positions and apparent molecular masses of the prestained protein standards are shown. The position of the 0th, 5th, 10th, 15th and 20th carbamylated carbonic anhydrase spots are indicated at the bottom of each gel.

pRE167



pRE167A103

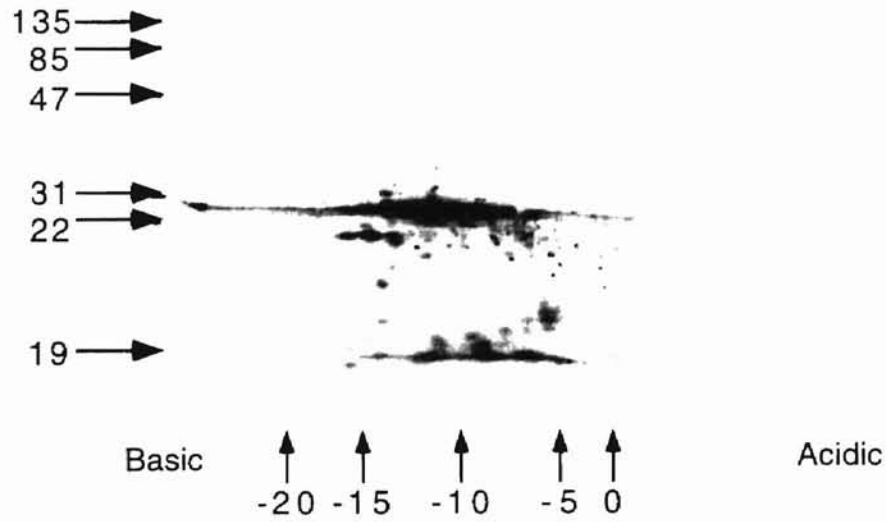
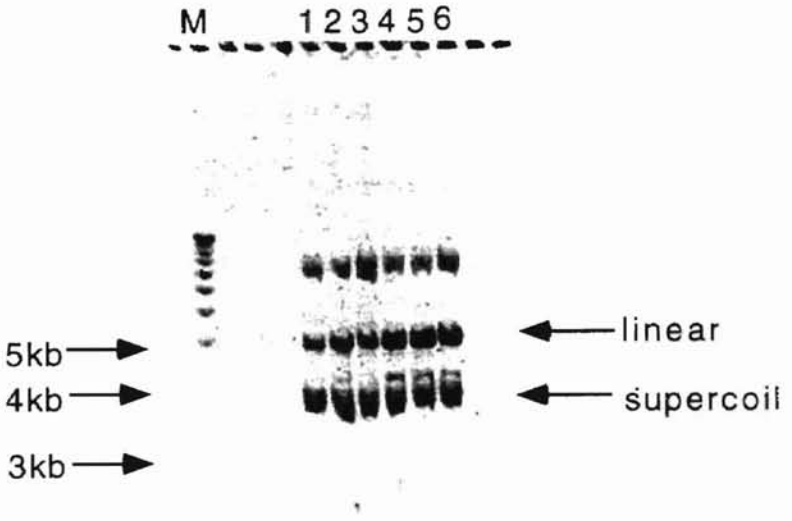


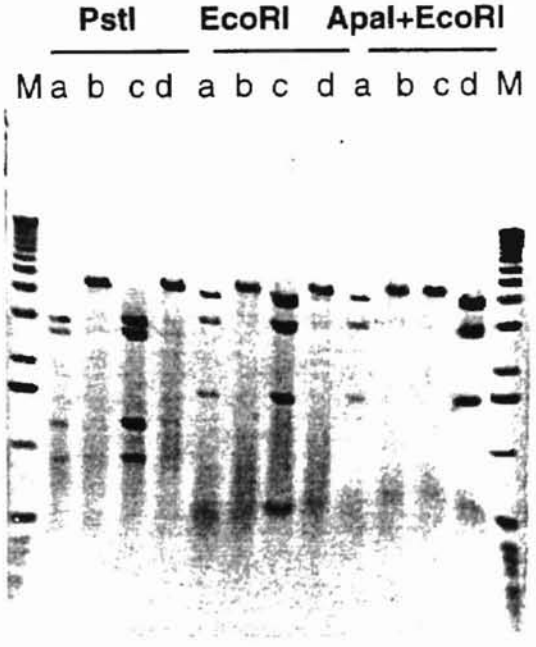
Fig. 16 Creation of replacement plasmid pTG105.

A. pRE167A10 partially digested with *Sma*I. M, 1 kb DNA ladder. Lane 1 to lane 6 are 0.5 ug pRE167A10 DNA digested with 0.2U, 0.3U, 0.35U, 0.4U, 0.45U, and 0.5U *Sma*I respectively. B. Plasmids isolated from kan^r and aro$^-$ colonies checked with restriction digestion. Plasmid a and c have the expected digestion pattern. c was designated as pTG105.

A.


M 1 2 3 4 5 6
5kb
4kb
3kb
linear
supercoil

B.


PstI
EcoRI
ApaI+EcoRI
M a b c d a b c d a b c d M

Chapter IV

Discussion

Over thirty genes are involved in the aromatic amino acid biosynthesis pathway in *Escherichia coli*. The *aroA* gene is highly conserved in the majority of species studied so far and often forms part of an operon with *serC*, the structural gene for 3-phosphoserine aminotransferase, with *serC* being proximal to the promoter for the operon and 70 bp upstream of *aroA* (Duncan and Coggins, 1986). In some bacteria, the *aroA* gene is in an operon with other genes, for example, *tyrA-aroA-aroK-pheA* in *Lactococcus lactis* (Griffin, 1995) and *aroC-aroB-aroA* in *Staphylococcus aureus* (O'Connell et al., 1993). However, *aroA* doesn't always form part of an operon: it has its own promoter in *Pasteurella haemolytica* serotype A1 (Tatum, 1994) and may be transcribed independently of *serC* in *Pasteurella multocida* (Homchampa, 1992). It is interesting that other genes in the aromatic amino acid biosynthesis pathway sometimes link together too, such as *aroF-tyrA-pheA* in *E.coli* (Hudson and Davidson, 1984), and a huge operon containing *aroC* (designated *aroF*)-*aroB-aroH-trp* operon-*hisH-tyrA-aroA* (designated *aroE*) in *Bacillus subtilis* (Henner et al., 1986 and O'Connell et al., 1993). The results of the present study also showed considerable homology between the deduced amino acid sequence of *B.abortus aroA* and that from other species (Fig. 13). The alignment indicated that translation may start from the second ATG codon because the sequence between the two initiation codons is not homologous to the sequences from other species. We don't think there is a *serC* gene upstream of the *aroA* in *B.abortus* since there is no

ORF in the 200 bp region upstream of the *aroA* gene, however, the nucleotide sequence of the 2.3 kb pRE167A10 showed another complete 558 bp open reading frame just downstream of *aroA* which has reasonable similarity with the cytidylate kinase sequence in other bacteria. These two are close enough that they should be in an operon. We didn't find any potential Shine-Dalgarno sequence or possible promoter sequence (-10 and -35 region) preceding either start codon. The aroA and the cytidylate kinase genes are in the same orientation as the beta-galactosidase gene of the pSK vector, so the expression of these pRE167 gene products may be under the control of the promoter from the vector or from *B.abortus* itself. We found a possible terminator sequence at the end of the cytidylate kinase gene which consists of a " stem-loop " structure with six-base stem, following by a poly adenylic acid tail (Fig. 11).

Ideally, the evolutionary relationships between organisms could be determined by comparing the nucleotide sequences of the respective genomes. However, this scale of analysis is generally impractical. Comparing sequences of individual genes also can give us information on evolutionary relationships between organisms. Small-subunit (16S or 18S) rRNA gene sequences are frequently used for gene-based phylogenetic classification schemes because of their slow rate of evolutionary changes. On our *aroA* phylogenetic trees, two major groups of bacteria could be seen. Proteobacteria of the gamma subdivision seem to cluster together except *D.nodosus* while the other subdivision , the gram-positive ones and the cyanobacteria distribute apart. The ones in operons didn't cluster together. This result is a little bit different from the 16S rRNA based tree which has the proteobacteria, gram-positive bacteria and cyanobacteria as three

separate groups, with the gram-positive one between the other two (Angert, 1996). This kind of exception also happened in phylogenetic trees of other Brucella genes (personal communication with Richard Essenberg). The sequence of a specific protein from bacteria sometimes may not represent the true evolutionary relationship because bacteria can take up exogenous DNA from other species via transformation, transduction or conjugation. It is not uncommon that a gene sequence appears in an unexpected position in the phylogenetic trees.

We constructed three phylogenetic trees by different methods and they appeared to be basically the same. The result from the Bootstrap program also showed that the trees are reliable because most branches appeared in a high percentage of the trees (Fig. 13 A).

Two major proteins were detected by the maxicell experiment. The 53 kDa one is consistent with the 1.4 kb *aroA* open reading frame in the nucleotide sequence and the aromatic amino acid requirement test. The 72 kD one is bigger than the protein expected from the 558 bp ORF next to the *aroA*. There are two possible reasons: first, there are actually three proteins translated from the 4.6 kb insert of pRE167: EPSP synthase, cytidylate kinase and the 72 kD protein. The 19 kDa cytidylate kinase may not show up well on the 2D-gel because of its very basic pI. The second possibility is that there are two proteins synthesized, the 53 kD EPSP synthase and 72 kD protein. The cytidylate kinase was not turned on in the maxicells for some unknown reasons. The 72 kDa protein being a fusion protein of the 53 kDa and 19 kDa proteins is not likely because of two things: first, pRE167A9 is a deletion plasmid deleted from the ApaI side but not into the

cytidylate kinase gene. It has lost the 72 kDa band but still has the 53 kDa band on the one-dimensional gel, showing that the 72 kDa protein is not a fusion protein. Another part of evidence is that the calculated pI of the 19 kDa cytidylate kinase is 9.26, but the pIs of the 72 kDa protein and the 53 kDa *aroA* proteins on the two-dimensional gel are acidic and neutral, respectively.

Although four replacement plasmids have been tried in the eletroporation experiment with *B.abortus* 2308, no potential mutants were found. Positive control pBBR was able to be introduced into the cells indicating that the electroporation system and the protocol works. Even single crossover events rarely happened because no kan resistant colonies were found to be amp resistant or sucrose sensitive for pTG103, pTG104 and pTG105. For pTG102, only one colony was kan resistant and sucrose sensitive, but no aro$^-$ mutant was obtained after the second selection. This may be explained by the low transformation frequencies of these replacement plasmids which were insufficient to routinely produce mutants by the rare event of homologous recombination or the suicide plasmids were degraded before they had a chance to get into the chromosome. It was reported by Tatum et al that an *aroA* mutant of *P. haemolytica* was produced by using a replicable but unstable replacement plasmid (Tatum, 1994). They repeated passage of *P.haemolytica* transformed with the replicable hybrid replacement plasmid to improve the chance of recombination and its instability allowed recovery of plasmidless *aroA* mutant. Another explanation for the unsuccessful result may be that the mutation is lethal. Although the transformation efficiency wasn't improved by supplementing the tryptose plates with 'aromix' after the electroporation, it is possible that disrupting the operon, but

not the *aroA* gene itself by a single crossover or a double crossover integration can be lethal and we didn't supplement the nutrient other than aromatic amino acids which *B.abortus* needs to survive.

The following further studies need to be done: get the whole sequence of pRE167 to identify the 72 kD unknown protein; use the deletions in the maxicell experiment to figure out where the 72 kDa protein is; improve the transformation efficiency by using different replacement plasmids to get higher recombination frequency. After we get the *aroA* deficient mutant, Southern blot analysis needs to be done to confirm the mutation. then macrophage cells will be used to test the virulence of the mutant.

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