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The Division and Cell Wall Gene Cluster of *Enterococcus hirae* S185

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Abstract

A chromosomal 10355-bp segment of *Enterococcus hirae* S185 contains nine *orfs* which occur in the same order as the MraW-, FtsL-, PBP3-, MraY-, MurD-, MurG-, FtsQ-, FtsA- and FtsZ-encoding genes of the division and cell wall clusters of *Escherichia coli* and *Bacillus subtilis.* The *E. hirae* DNA segment lacks the genes which in *E. coli* encode the ligases Ddl, MurC, MurE and MurF and the integral membrane protein FtsW. The encoded E. *hirae* and *E. coli* proteins share 25 % to 50 % identity except FtsL and FtsQ (s 14 % identity).

Keywords : *dcw* gene cluster ; *Enterococcus hirae* ; *Escherichia coli* ; *Bacillus subtilis* ; cell septation

The *dcw* (*d*ivision and *c*ell *w*all) cluster at the 2-min region of the chromosome of *Escherichia coli* contains genes the products of which are involved in the bacterial cell wall peptidoglycan synthesis and assembly (For primary references, see Yura *et al.*, 1992 ; van Heijenoort, 1994 ; Ayala *et al.*, 1994 ; Ghuysen et *al.*, 1996 ; Vicente and Errington, 1996). Five ligases, Ddl, MurC, MurD, MurE and MurF, catalyse the formation of D-alanyl-D-alanine (Ddl) and the sequential addition to UDP-N-acetylmuramic acid of L-alanine (MurC), D-glutamic acid (MurD), *meso*-diaminopimelic acid (MurE) and the preformed D-alanyl-D-alanine (MurF), achieving the synthesis of the nucleotide precursor UDP- N-acetylmuramoyl-L-alanine-γ-D-glutamyl-(L) *meso*-diaminopimelyl-D-alanyl-D-alanine. The transphosphorylase MraY transfers the phospho-N-acetylmuramoyl-pentapeptide moiety of the uridylic acid carrier to the transmembrane C_{55}-isoprenoid alcohol phosphate, and the transglycosylase MurG transfers the N-acetylglucosamine residue of UDP-N-acetylglucosamine to the N-acetylmuramoyl moiety of the lipid precursor, achieving the synthesis of the lipid-linked disaccharide (N-acetylglucosaminyl-N-acetylmuramoyl)-pentapeptide, or lipid II intermediate. The multimodular class B penicillin-binding protein (PBP)3 and several non-penicillin-binding proteins target the wall peptidoglycan assembly machinery to septum formation. PBP3 (the penicillin-binding module of which is an acyl serine transferase), FtsL (a protein with a putative leucine zipper motif) and FtsQ are membrane-bound with the bulk of the polypeptide chains exposed in the periplasm. FtsW is an integral membrane protein with loops exposed on both sides of the membrane. FtsA, an isologue of the DnaK-actin family of ATPases, is cytosolic when phosphorylated and membrane-associated when unphosphorylated. MraW, a protein with a putative S-adenosyl-methionine-binding motif, and FtsZ, a GTPase that has similarity to tubulin, are cytosolic. FtsZ functions as a cytoskeletal element mediating the invagination of the septum.

The *Bacillus subtilis dcw* cluster is located at the 130°-135° region of the chromosome (Buchanan *et al.*, 1994 ; Kunst *et al.*, 1997). It contains genes encoding proteins analogous to the *E. coli* ligases MurD and MurE, the transphosphorylase MraY, the transglycosylase MurG, the multimodular class B PBP3 (*i.e*. PBP2b and SpoVD) and the cell cycle proteins MraW, FtsL, FtsW, FtsQ, FtsA and FtsZ. The *B. subtilis* cluster, however, lacks the ligase-encoding genes *ddl*, *murC* and *murF* and it contains additional genes related to sporulation.

The high-molecular-mass PBP3s of *Enterococcus hirae* (the suffix « s » denotes the susceptibility of this PBP to β-lactam antibiotics in opposition to the low affinity of PBP3r) was known to be involved in cell septation (Coyette *et al.*, 1983). It was expected to be the counterpart of *E. coli* PBP3 and *B. subtilis* PBP2b and its encoding gene was expected to be part of a *dcw* cluster. To check the validity of the hypothesis, a 10355-bp DNA segment of *E. hirae* S185, containing the PBP3s-encoding gene, was sequenced and analysed.

The *E. hirae* genomic DNA was isolated (Hopwood *et al.*, 1985) from cells grown unshaken at 37°C in Brain-Heart medium and collected at the end of the exponential phase. Restricted fragments were cloned into pUC18 or pUCBM20 and the inserts were sequenced on both strands using the T7 sequencing kit with [^{35}S]dATP labelling, and the Autoread or ThermoSequenase sequencing kits with 5'-fluorescein or Cy5 primers, in which case the electrophoresis was performed on an ALF express DNA sequencer. The nucleotide sequences were introduced in GELASSEMBLE (Pearson *et al.*, 1988), the ORFs were identified with CODON PREFERENCE (Devereux *et al.*, 1984) and homology searches (SWISS-PROT, PIR, Genpept) were made by using FASTA or BLASTP

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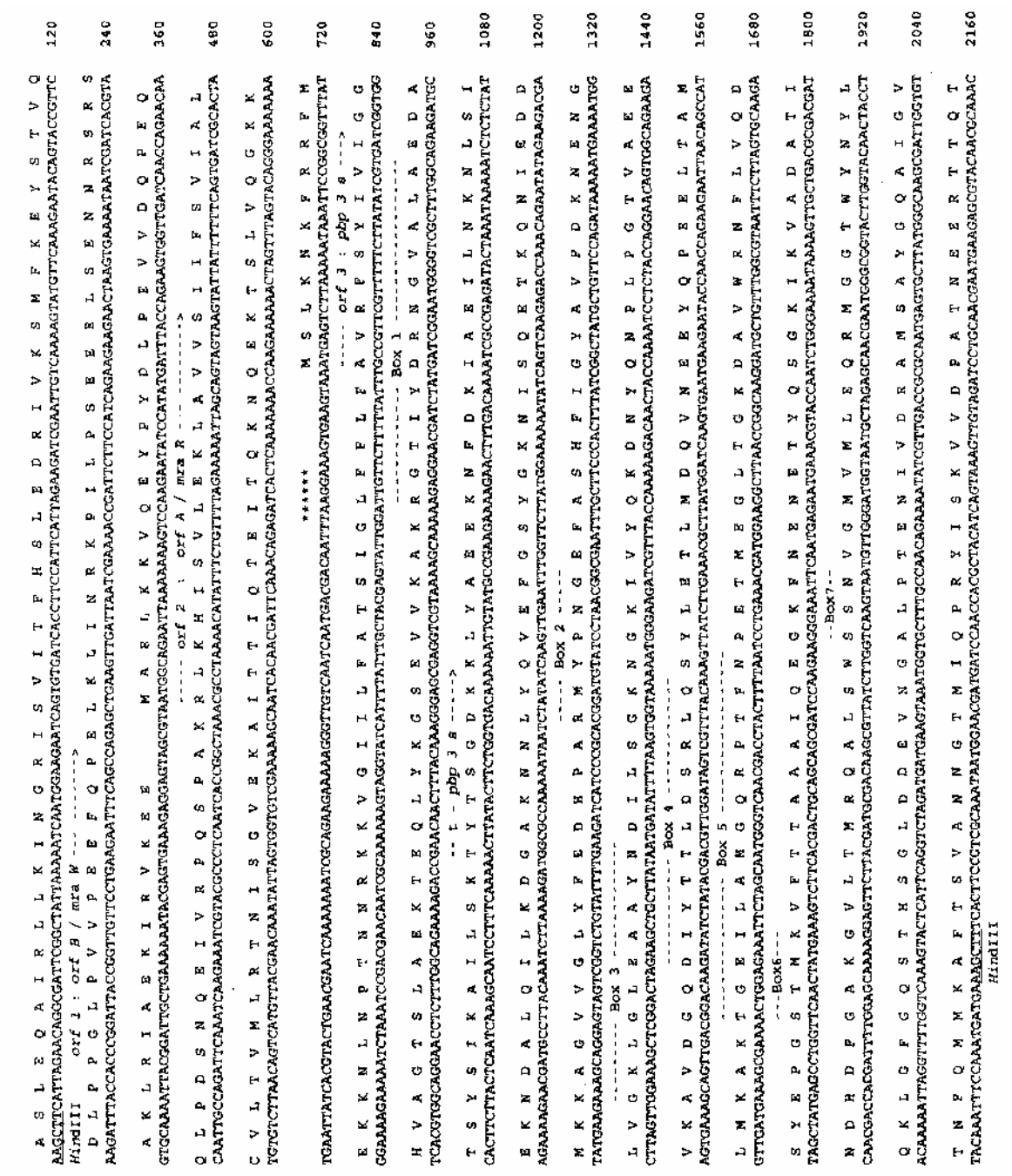
(Altschul *et al.*, 1990).

The results of these studies are summarized in Figs 1 and 2. Fig. 1 gives the nucleotide sequence of the 10355-bp segment of *E. hirae* S185 and it translates the *orfs* into amino acid sequences. Fig. 2 compares pair-wise the proteins encoded by the *dcw* clusters of *E. hirae*, *E. coli* and *B. subtilis.*

The E. *hirae dcw* cluster was identified using a six-step strategy. The nucleotides and amino acid residues mentioned below refer to Fig. 1.

FIGURE 1 Nucleotide and deduced amino acid sequences of the E. hirae S185 *dcw* ***cluster.*** *Putative ribosome-binding sites are indicated by asterisks above the nucleotide sequence. Motifs (boxes) 1-9 of PBP3s, characteristic of the multimodular class B PBPs (Ghuysen, 1997), are indicated above the amino acid sequence. t-pbp3s : tryptic fragment-encoding sequence (Piras et al., 1990). Nucleotide sequence accession number : Y13922 (EMBL data bank)*



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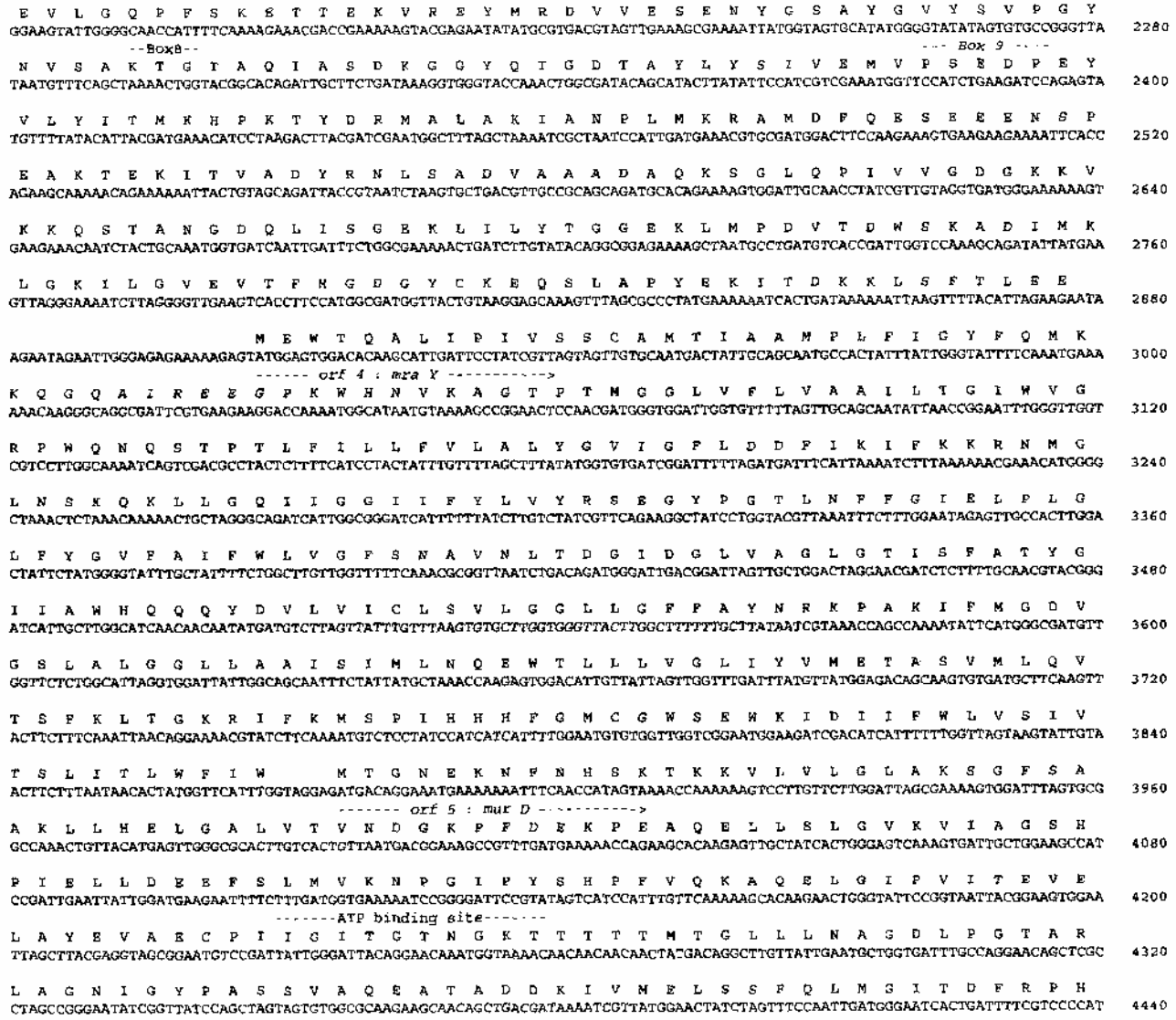

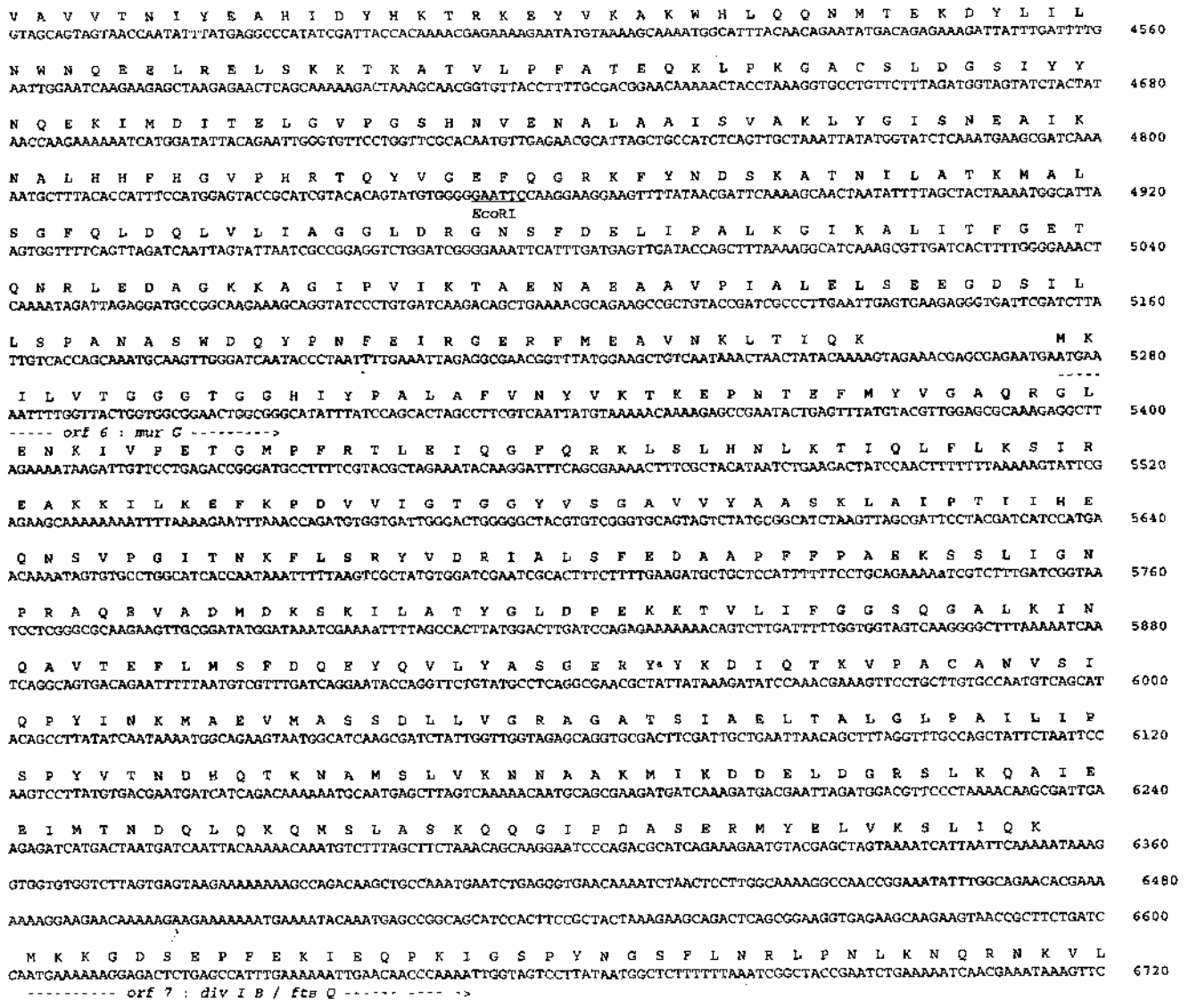
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N W N Q E E L R E L S K K T K A T V L P F A T E Q K L P K G A C S L D G S I Y Y
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EcoRI
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----- orf 6 : mur G ---------->
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P R A Q E V A D M D K S K I L A T Y G L D P E K K T V L I F G G S Q G A L K I N
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---------- orf 7 : div I B / fts Q ------ ---- ->
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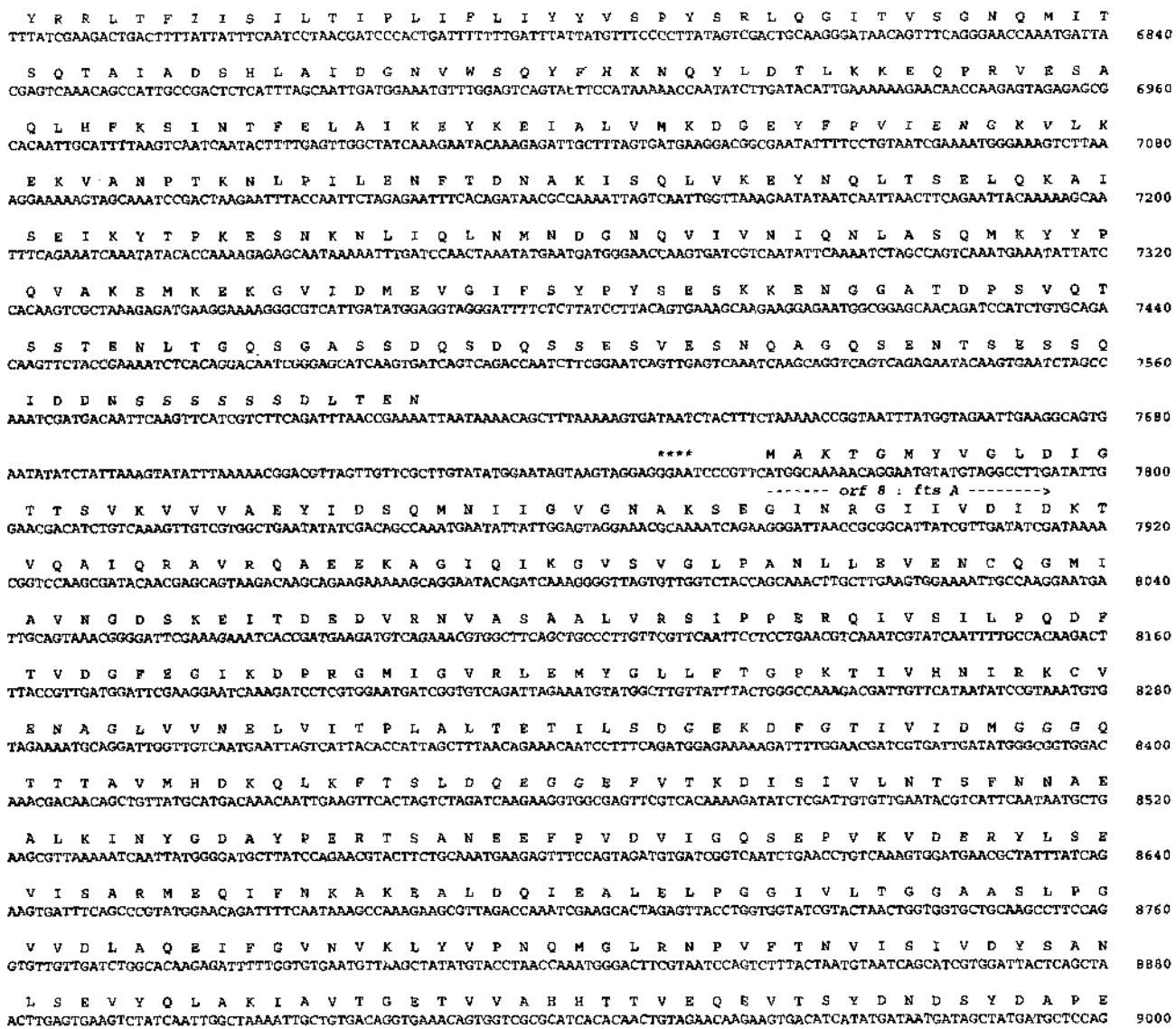

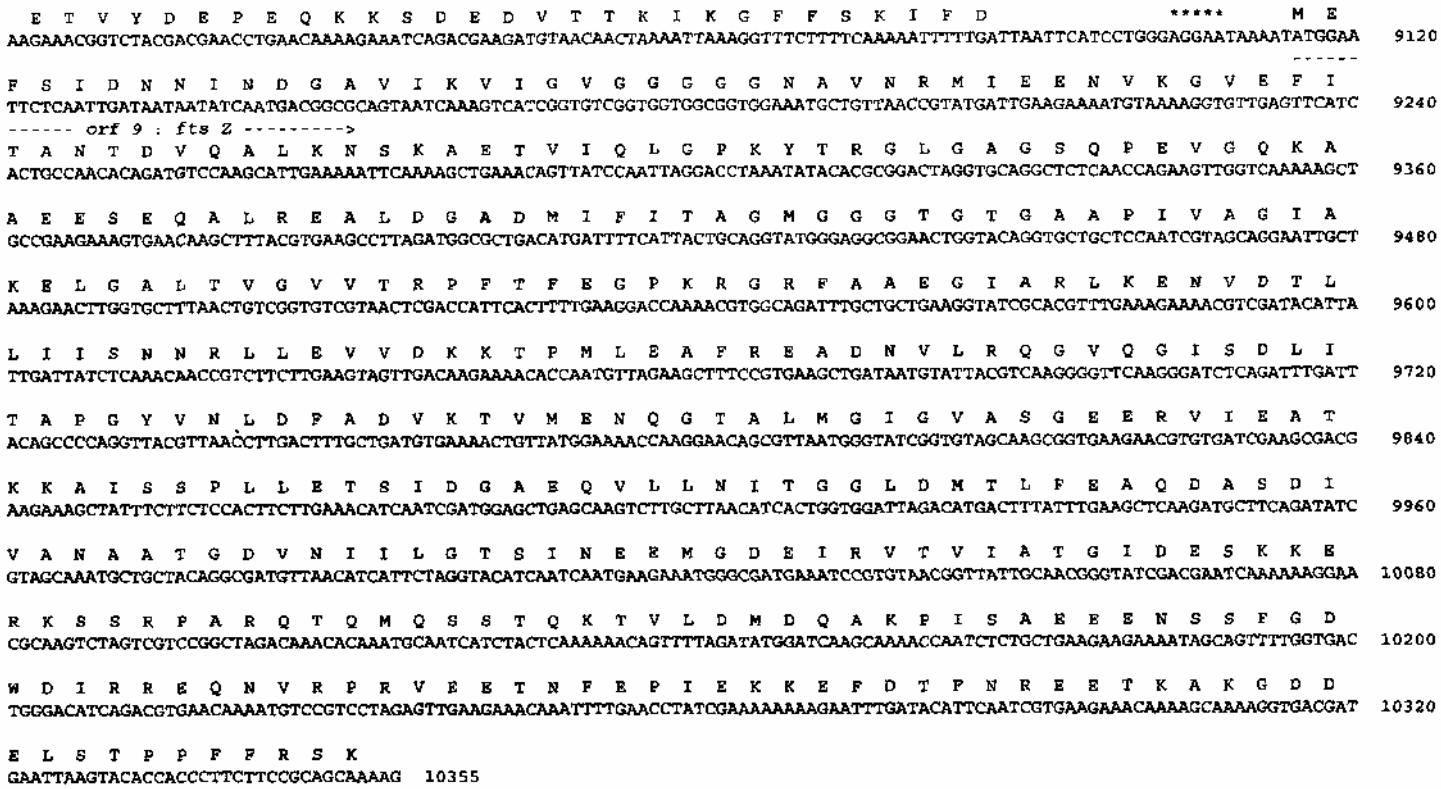

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Step1. The primers

5′GAGGCATGCACIGGICAACTITACAAGGG3′
*Sph*I GT T A

and

5′CCCGAGCTCACATAIGAIGTIGCGTCCTC3′
*Sac*I G CT A T

(with I denoting inosine) were synthesized on the basis of the sequence of the amino terminal region (37 residues) of a 58-kD tryptic fragment of *E. hirae* PBP3s (Piras *et al.*, 1990). They allowed a 119-bp DNA segment of the genomic DNA to be amplified by PCR. The reaction product generated by the Taq and Dynazyme polymerases each encoded the polypeptide T62-(S)95, the sequence of which was that of the amino-terminal end of the tryptic fragment except that E occurred at position 63 instead of G and S occurred at position 95 instead of V.

Step 2. The primer

5′GAGGGTACCATCGTTC–
*Asp*718

CTCTTTTTGCTTTTACG3′

the 24 last nucleotides of which were complementary to nt 924 - nt 901, and the primer

5′CCCGAGCTCCTATGATCGGAATGGGGTCG3′
*Sac*I

the 20 last nucleotides of which corresponded to nt 925 - nt 944 were used in inverse PCRs (Silver *et al.*, 1991) carried out on *Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Hind*III and *Xba*I genomic libraries. The 2-kb DNA fragments amplified from the *Hind*III library by the Taq and Dynazyme polymerases each were digested with *Hind*IΠ and *Asp*718, and with *Hind*IΠ and *Sac*I. The inserts were cloned and sequenced, yielding the sequence nt 1 - nt 2066.

Step 3. The primer

5′CCCGGTACCGAAGGTG–
*Asp*718

ATCACACTGATTCTTCC3′

the 24 last nucleotides of which were complementaryto nt 65 - nt 42 and the primer

5′GAGGAGCTCCGACAAGCGTTATCTTGG3′
*Sac*I

the 18 last nucleotides of which corresponded to nt 1841 - nt 1858, were used in inverse PCRs carried out on *Bgl*II, *Eco*RI and *Hind*III genomic libraries using the Goldstar polymerase. A 5.5-kb DNA fragment of the *Eco*RI library was digested with *Sac*I and *Eco*RI. Sequencing of the released 3-kb DNA fragment allowed several *orfs* to be identified.

*orf*1 (nt 1 - nt 287; truncated at the 5' end) encoded a 94 amino acid residue polypeptide similar to the carboxy terminal region of the *E. coli* MraW (38 % identity) and *B. subtilis* ORFB (72 % identity). *orf*2 (nt 292 - nt 603) encoded a 103 amino acid residue protein weakly related to the *E. coli* MraR/FtsL (14 % identity) and *B. subtilis* ORFA (21 % identity). *orf*3 (nt 689 - nt 2878) encoded the 730 amino acid residue multimodular class B PBP3s similar to the 588 amino acid residue *E. coli* PBP3 (27 % identity) and the 716 amino acid residue *B. subtilis*

PBP2b (36 % identity). Most class B PBPs, including *E. coli* PBP3, terminate 60-90 residues downstream from the KTGTA motif of the acyl serine transferase-penicillin-binding module. In contrast, *B. subtilis* PBP2b and SpoVD, and *E. hirae* PBP3s each bear a carboxy terminal extension, 150-200 amino acid residues long. *orf*4 (nt 2908 - nt 3873) encoded a 321 amino acid residue protein similar to the *E. coli* and B. *subtilis* MraY (43 % and 47 % identity). *orf*5 (nt 3877 - nt 4857) was truncated at the 3' end.

Step 4. In analogy with *E. coli* and *B. subtilis, E. hirae* was expected to possess a *ftsW*/*rodA*-like gene downstream from *murD* (*orf5*). Consequently the primer

5′CCCGAGCTCGCTTTACACCATTTCCATGG3′
*Sac*I

the 20 last nucleotides of which corresponded to nt 4804 - nt 4823 and the primer

5′GAGGGTACCGGTCAATACAATTACGCCC3′
*Asp*718

the 19 last nucleotides of which were complementary to the G77VIVLT82-encoding sequence of the *E. hirae* FtsW (unpublished data) were used in a direct PCR carried out on genomic DNA. Sequencing the 2.6-kb product generated by the Goldstar polymerase allowed the 3' portion of *orf*5 (nt 4858 - nt 5259) to be completed and two additional *orfs* to be identified.

*orf*5 (nt 3877 - nt 5259) encoded a 460 amino acid residue protein similar to the *E. coli* and B. *subtilis* MurD (33 % and 51 % identity). *orf*6 (nt 5276 - nt 6358) encoded a 360 amino acid residue protein similar to the *E. coli* and *B. subtilis* MurG (32 % and 52 % identity). *orf*7 (nt 6603 - nt 7350; truncated at the 3' end) encoded a polypeptide which had similarity with the amino terminal region of the *B. subtilis* DivIB (Harry *et al.*, 1989) and *E. coli* FtsQ.

Step 5. In analogy with *E. coli* and *B. subtilis, E. hirae* was expected to possess *ftsA*- and *ftsZ*-like genes downstream from *ftsQ*. The FtsZ sequences of *E. coli*, *B. subtilis* and some other bacteria each possess the hexapeptide GADMVF (Margolin *et al.*, 1996). On this basis, the primer

5′GAGGGTACCAAIACCATGTCIGCICC3′
*Asp*718 A

the 17 last nucleotides of which were complementary to the hexapeptide-encoding sequence and the primer

5′CCCGAGCTCATATGAAT–
*Sac*I

GATGGGAACCAAGTGA3′

the 24 last nucleotides of which corresponded to nt 7252 - nt 7275 were used in a direct PCR carried out on genomic DNA with the Dynazyme polymerase. Sequencing the 2.1-kb product allowed the 3' portion of *orf*7 (nt 7351 - nt 7610) to be completed and two additional *orfs* to be identified.

*orf*7 (nt 6603 - nt 7610) encoded a 335 amino acid residue protein weakly related to *E. coli* FtsQ (14 % identity) but significantly similar to *B. subtilis* DivIB/FtsQ (33 % identity). *orf*8 (nt 7764 - nt 9092) encoded a 442 amino acid residue protein similar to the *E. coli* and *B. subtilis* FtsA (33 % and 39 % identity). *orf*9 (nt 9115 - nt 9416; truncated at the 3'end) encoded a 100 amino acid residue polypeptide similar to the amino-terminal region of the *E. coli* and *B. subtilis* FtsZ.

Step 6. On the basis of the known *E. faecalis* FtsZ-encoding gene (accession n°U94707), the degenerated primer

5′CGCTTICGICGGAAGAAIGG3′
TT T TA A

(with I denoting iosine) complementary to the sequence encoding the peptide PFFRRK(R) which occurs at the carboxy end of the protein, and the primer 5'GGACTAGGTGCAGGCTCTC AACC3' corresponding to nt 9313 - nt 9341, were used in a direct PCR carried out on genomic DNA with the Taq DNA polymerase. Sequencing of the amplified 1043-bp DNA fragment allowed *orf*9 to be completed. This *orf* (nt 9115 - nt 10355) encoded a protein at least 413 amino acid residues long, similar to the *E. coli* and *B. subtilis* FtsZ (50 % and 63 % identity). One may note that because of the degenerated primer used, the 17 nucleotide sequence at the 3' end of the 10355-bp segment may not be accurate but the encoded amino acid residues are likely to be exact.

The *dcw* cluster shown in Fig. 2 is that of *E. hirae* strain S185. All the genes are oriented in the same direction of transcription and they do not overlap. PCRs were also carried out on the genomic DNAs of *E. hirae* S185, *E. hirae* ATCC9790 and *E. hirae* R40 using as primers the sequences nt 252-273 and nt 1620-1597 (pair 1), nt 1278-1301 and nt 3759-3737 (pair 2), nt 3737-3759 and nt 6456-6434 (pair 3), nt 5210-5231 and nt 7364-7346 (pair 4) and nt 7255-7278 and nt 8177-8148 (pair 5). Consistent with the patterns of the reaction products, the three *E. hirae* strains are expected to have similar or identical *dcw* clusters. Likewise, the *dcw* cluster of *E. faecalis* has exactly the same organization as that of *E. hirae* and the encoded proteins are very homologous ; the *Staphylococcus aureus dcw* cluster is also very similar except that it lacks *murG* (Pucci *et al.*, 1997).

In *E. coli*, *mrdB* which encodes the integral membrane protein RodA (which is very similar to FtsW) is located outside the *dcw* cluster at the 14-min region of the chromosome. Likewise, *a ftsW*/*rodA*-like gene is not present in the 10355-bp DNA segment of *E. hirae*. The gene, however, has been identified in plasmid pDML540 upstream from *psr*, itself located upstream from the low-affinity PBP5-encoding gene (unpublished results).

dcw clusters are likely to be ubiquitous in the bacterial world but with species-specific variations. The *E. coli dcw* cluster (see the Introduction) contains the complete set of genes that encode the ligases Ddl, MurC, MurD, MurE and MurF involved in the conversion of UDP-N-acetylmuramic acid into UDP-N-acetylmuramoyl-pentapeptide. The *B. subtilis dcw* cluster lacks the Ddl- and MurC-encoding genes and the *E. hirae dcw* cluster lacks the Ddl-, MurC-, MurE- and MurF-encoding genes. However, the *E. coli*, *B. subtilis* and *E. hirae dcw* clusters, each contain the genes that encode the MraY transphosphorylase and the MurG transglycosylase involved in the synthesis of the lipid II intermediate, which is the immediate precursor used for wall peptidoglycan assembly. They each also contain the genes that encode MraW, FtsL, PBP3 (PBP2B/SpoVD), FtsQ (DivIB), FtsA and FtsZ, which are essential components of the cell septation network.

These cell division proteins are widespread in the bacterial world and most of them are much conserved (Table I). The percentages of identity relative to the *E. hirae* proteins are greater than 24 % except for the *E. coli* FtsQ (14 % identity). FtsZ which polymerizes to form a circumferential ring at the division site, is also present in mycoplasma (Wang and Lutkenhaus, 1996) which are wall-less eubacterial organisms and in archaeobacteria. Interestingly, phylogenetic trees consistently place the archaeobacterial FtsZ closer to the eukaryotic tubulins relative to the eubacterial FtsZ proteins (Margollin *et al.*, 1996).

TABLE I Proteins isologous to the E. hirae PBP3s, MraY, MurG, FtsQ, FtsA and FtsZ. *The percentages of amino acid similarity and identity were calculated with the BESTFIT algorithm. The proteins are listed in decreasing order of identity relative to the corresponding E. hirae proteins*

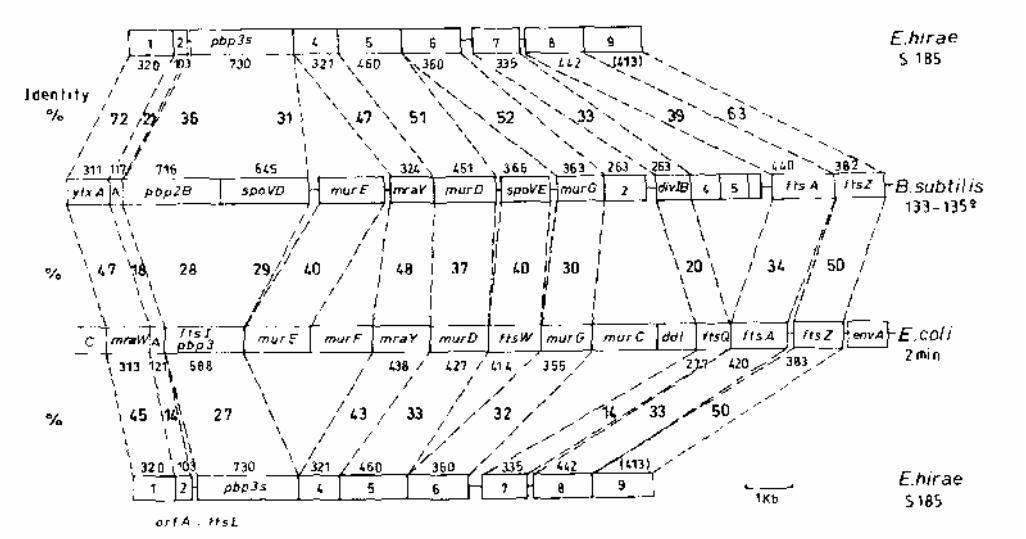
	Accession number	***Protein***	***Similarity***	***Identity***
PBP3s				
Enterococcus faecalis	U94707[x]	PBPC	75.5	59.3
Streptococcus mitis	X78216[x]	PBPX	62.5	42.7
Streptococcus oralis	X78217[x]	PBPX	61.5	42.5
Streptococcus pneumoniae	X78215[x]	PBPX	61.1	41.6
Streptococcus pneumoniae	P14677[o]	PBP2X	60.6	41.3
Bacillus subtilis	Q07868[o]	PBP2B	56.7	36.3
Staphylococcus aureus	D28879[x]	PBP1	55.0	33.2
Bacillus subtilis	Q03524[o]	SpoVD	52.4	31.1
Pseudomonas aeruginosa	X95517[x]	PBP3A	50.2	29.5
Haemophilus influenzae	P45059[o]	PBP3	48.3	29.0
Pseudomonas aeruginosa	S54872[+]	PBP3	48.7	28.1
Neisseria sicca	X76285[x]	PBP2	49.7	27.2
Neisseria gonorrhoeae	P08149[o]	PBP2	49.6	27.2
Neisseria meningitidis	S49098[+]	PBP2	49.4	27.1

Escherichia coli	P04286[o]	PBP3	48.7	27.0
Bacillus subtilis	P42971[o]	hypothetical 74.4 kD prot.	48.7	26.5
Enterococcus hirae R40	X62280[x]	PBP5	47.4	26.4
Escherichia coli	P08150[o]	PBP2	48.8	26.3
Helicobacter pylori	HP1565[•]	PBP2	48.0	26.3
Enterococcus hirae	A36903[+]	PBP3r	49.6	25.9
Enterococcus faecium	X92687[x]	PBP5	49.2	25.2
Helicobacter pylori	HP1556[•]	FtsI	47.6	24.3
MraY				
Enterococcus faecalis	U94707[x]	MraY	90.9	72.8
Staphylococcus aureus	U94706[x]	MraY	74.8	50.7
Bacillus subtilis	Q03521[o]	MraY	73.7	46.8
Escherichia coli	P15876[o]	MraY	68.6	43.4
Haemophilus influenzae	A64185[+]	MraY	67.4	42.8
Borrelia burgdorferi	X96432[x]	MraY	68.8	42.8
Synechocystis sp.	D64005[x]	MraY	63.9	40.0
Staphylococcus aureus	A55856[+]	L1m[1]	60.6	30.5
Escherichia coli	P24235[o]	Rfe[2]	58.9	28.7
Mycobacterium leprae	P45830[o]	Rfe homolog	57.3	28.3
Pseudomonas aeruginosa	U17293[x]	Rfb303[3]	58.6	27.7
Methanococcus jannaschii[5]	U67554[x]	Diaminopimelate epimerase	57.2	27.3
Yersinia enterocolitica	S51265[+]	TrsF[4]	58.6	26.7
Haemophilus influenzae	A64138[+]	Rfe homolog	54.5	25.0
MurG				
En terococcus faecalis	U94707[x]	MurG	79.3	66.8
Bacillus subtilis	P37585[o]	MurG	71.0	52.1
Haemophilus influenzae	P45065[o]	MurG	53.3	32.0
Escherichia coli	P17443[o]	MurG	57.2	31.7
FtsQ				
Enterococcus faecalis	U94707[x]	DivIB	57.4	39.4
Bacillus licheniformis	U01958[x]	DivIB	54.1	33.7
Bacillus subtilis	P16655[o]	DivIB	52.5	32.8
Staphylococcus aureus	U94707[x]	DivIB	50.3	28.9
Escherichia coli	K02668[x]	FtsQ	38.1	14.3
FtsA				
Enterococcus faecalis	U94707[x]	FtsA	87.8	76.2
Bacillus subtilis	P28264[o]	FtsA	63.6	39.1
Borrelia burgdorferi	Z12164[x]	FtsA	56.6	32.6
Escherichia coli	P06137[o]	FtsA	56.9	33.0
Haemophilus influenzae	P45068[o]	FtsA	57.4	30.9
Sinorhizobium meliloti	Af024660[x]	FtsA	54.8	30.2
Staphylococcus aureus	U94706[x]	FtsA	52.0	28.8
Helicobacter pylori	HP0978[•]	FtsA	49.0	27.0
FtsZ				
Enterococcus faecalis	U94707[x]	FtsZ	88.5	82.2
Bacillus subtilis	P17865°	FtsZ	75.9	62.9
Staphylococcus aureus	U94706[x]	FtsZ	74.3	59.7
Anabaena sp	JC4289[+]	FtsZ	68.3	55.3
Streptomyces coelicolor	P45500[o]	FtsZ	68.4	52.2
Synechocystis sp	P73456[o]	FtsZ	68.2	51.3
Borrelia burgdorferi	P45483[o]	FtsZ	70.8	50.9
Brevibaclerium lactofermentum	P94337[o]	FtsZ	68.0	50.9
Corynebacterium glutamicum	Ab003132[x]	FtsZ	68.7	50.5
Mycoplasma pulmonis	Q50318[o]	FtsZ	68.1	50.4
Pseudomonas aeruginosa	P47204[o]	FtsZ	66.4	50.3
Escherichia coli	P06138[o]	FtsZ	70.1	50.1

Streptomyces griseus	P45501[o]	FtsZ	65.1	49.6
Neisseria meningitidis	U43329[x]	FtsZ	67.6	49.3
Thermotoga maritima	U65944[x]	FtsZ	71.5	46.9
Azotobacter vinelandii	P77817[o]	FtsZ	65.4	46.7
Neisseria gonorrhoeae	P72079[o]	FtsZ	66.4	46.7
Bartonella bacilliformis	Af007266[x]	FtsZ	64.4	45.9
Pseudomonas putida	U29400[x]	FtsZ	64.6	45.8
Agrobacterium tumefaciens	Af024659[x]	FtsZ	64.7	45.6
Haloferax volcanii[5]	U37584[x]	FtsZ	67.8	45.1
Wolbachia sp	P45485[o]	FtsZ	64.7	45.0
Pyrococcus woesci[5]	U56247[x]	FtsZ	64.6	45.0
Haemophilus influenzae	P45069[o]	FtsZ	67.2	44.6
Rhizobium meliloti	M94386[x]	FtsZ	61.9	44.4
Caulobacter crescentus	P52976[o]	FtsZ	61.2	44.2
Methanococcus jannaschii[5]	Q58039[o]	FtsZ	64.6	43.7
Helicobacter pylori	HP0979[•]	FtsZ	62.2	41.4
Halobacterium salinarium[5]	U32860[x]	FtsZ	60.0	37.1
Mycoplasma genitalium	P47466[o]	FtsZ	46.9	25.1
Mycoplasma pneumoniae	P75464[o]	FtsZ	52.0	24.2

[1]Llm: protein affecting the methicillin resistance level and the autolysis rate in *S. aureus.*
[2]Rfe: putative undecaprenyl-phosphate α-N-acetylglucosaminyl transferase.
[3]Rfb303: B-band lipopolysaccharide biosynthesis protein.
[4]TrsF: protein involved in lipopolysaccharide core biosynthesis.
[5]*M. jannaschii*, *H. volcanii*, *P. woesci* and *H. salinarium* are archaeobacteria.
[+]: PIR bank ; [o]: SWISSPROT bank ; [x]: EMBL/GENbank/DDBJ; [•]: TIGR databank.

FIGURE 2 The dcw cluster of E. hirae S185, E. coli and B. subtilis. *Genes are boxed. Numbers of amino acid residues of the encoded proteins are given below and above the genes. Similarity between pairs of amino acid sequences is expressed in percent identity*



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