Microbiology (1998), 144, 3069–3078 Printed in Great Britain

Unconventional organization of the division and cell wall gene cluster of *Streptococcus pneumoniae*

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The genes responsible for cell wall biosynthesis and cell division (*dcw* genes) were identified and sequenced in *Streptococcus pneumoniae*. The genetic organization of the *dcw* cluster in *Streptococcus pneumoniae* differed significantly from the clusters of other bacteria reported to date. In particular, the genes corresponding to the 2 min region of the *Escherichia coli* chromosome were found distributed in three genetically separate regions of the *Streptococcus pneumoniae* chromosome. The first region contained the expected *ftsA* and *ftsZ* cell division genes at one end and *pbp2b*, *ddl* and *murF* at the other end. The *murD*, *murG* and *divIB* genes, always found located upstream of *ftsA*, were found in a second region separated from the first. A third region contained the *yllC*, *yllD*, *pbp2x* and *mraY* genes. The chromosomal region downstream of *ftsZ* was also sequenced and characterized. In *Streptococcus pneumoniae* this region contains four ORFs, all of unknown function, and an ORF encoding the *Bacillus subtilis* DivIVA homologue. The gene order and the organization of this region was found to be conserved in *Staphylococcus aureus*, *Streptococcus pyogenes* and *Bacillus subtilis*, raising the possibility that previously unidentified loci may also be involved in division.

Keywords: *dcw* gene cluster, *Streptococcus pneumoniae*, *Staphylococcus aureus*, cell division, Gram-positive bacteria

INTRODUCTION

Many different bacteria, including Gram-negative and Gram-positive rods or cocci, possess a chromosomal region similar to that found at the 2 min region of the *Escherichia coli* chromosome (Yura *et al.*, 1992; Ayala *et al.*, 1994). This region contains many genes whose products are involved in division and cell wall biosynthesis and is therefore known as the *dcw* (division and cell wall) cluster (Ayala *et al.*, 1994; Vicente & Errington, 1996).

Initial comparison between *Escherichia coli* and *Bacillus subtilis dcw* clusters showed a remarkable degree of conservation (Henriques *et al.*, 1992; Buchanan *et al.*, 1994), not only at the level of sequence similarity among the translated sequences, but also in the organization of the genes and the polarity of their transcription. Both regions contain a *pbp* gene (*ftsI*/*pbpB*) near the beginning of the cluster and *ftsQ* (*divIB*), *ftsA* and *ftsZ*, next to each other, at the distal end. In *Bacillus subtilis*, *pbpB*, encoding PBP2B (Yanouri *et al.*, 1993), is duplicated and its homologue, *spoVD*, has been shown to be expressed differentially during sporulation (Daniel *et al.*, 1994). Three additional members of the cluster, *yllB*, *yllC* and *yllD*, located upstream of *pbpB*, have been reported in *Bacillus subtilis* (Daniel *et al.*, 1996). Putative structural or functional homologues of these genes, *mraZ*, *mraW* and *mraR* (now named *yabB*, *yabC* and *ftsL*, respectively), are present in the same location in the *Escherichia coli* chromosome and are believed to play similar roles in division (Ayala *et al.*, 1994; Daniel *et al.*, 1996).

Currently, the sequence and the organization of the *dcw* clusters of *Haemophilus influenzae* (Fleischmann *et al.*,

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The GenBank accession numbers for the sequences reported in this paper are AF068901, AF068902 and AF068903 for *Streptococcus pneumoniae* and AF068904 for *Staphylococcus aureus*.

1995; Lutkenhaus & Addinall, 1997), *Staphylococcus aureus* (Pucci *et al.*, 1997), *Enterococcus faecalis* (Pucci *et al.*, 1997) and *Enterococcus hirae* (Duez *et al.*, 1997) are available. While the *Haemophilus influenzae dcw* cluster is essentially identical to that of *Escherichia coli*, some differences were found in the comparison of this region to that of Gram-positive bacteria. Some genes encoding enzymes involved in the cytoplasmic step of peptidoglycan biosynthesis are not included in the cluster of Gram-positive bacteria (Buchanan *et al.*, 1994; Pucci *et al.*, 1997; Duez *et al.*, 1997). In particular, the *Bacillus subtilis* cluster does not contain the equivalent of *murF*, *murC* and *ddl* (Buchanan *et al.*, 1994). Consistently, these genes are missing, together with *murE*, in *Enterococcus faecalis*, *Enterococcus hirae* and *Staphylococcus aureus*, which also lacks *murG* (Pucci *et al.*, 1997; Duez *et al.*, 1997). However, since these genes are believed to be essential, they are expected to be placed in chromosomal locations other than the *dcw* cluster. Interestingly, *ftsW*, which encodes an essential protein required at the early and late stages of division (Ishino *et al.*, 1989; Khattar *et al.*, 1994, 1997), is also missing from the *dcw* cluster of all the Gram-positive bacteria analysed so far. However, a sporulation-specific locus, the *ftsW* homologue *spoVE*, has been found between *murD* and *murG* in *Bacillus subtilis* (Ikeda *et al.*, 1989; Henriques *et al.*, 1992; Daniel & Errington, 1993). Moreover, a gene encoding an FtsW-like protein has been found in *Staphylococcus aureus* (Pucci *et al.*, 1997) and in *Enterococcus hirae* (O. Massidda, C. Duez, J. Coyette, G. D. Shockman & L. Daneo-Moore, unpublished results; GenBank accession no. U58049) and in all cases mapped elsewhere in the chromosome.

The presence of the *dcw* cluster in several different bacterial species has been interpreted to have regulatory and evolutionary implications (Vicente & Errington, 1996; Hara *et al.*, 1997; Watanabe *et al.*, 1997). In this work we report the sequence and organization of the *dcw* cluster of *Streptococcus pneumoniae*. We found that the organization of the *dcw* genes differs in many respects from that of the clusters of other Gram-positive and Gram-negative bacteria reported so far. We also report the nucleotide sequence of a 3·1 kb chromosomal region identified downstream of *ftsZ*. This region contains four ORFs encoding proteins of unknown function, followed by an ORF encoding the *Bacillus subtilis* DivIVA homologue, recently found to play a role in division (Cha & Stewart, 1997; Edwards & Errington, 1997). An *ftsZ–divIVA* intergenic region could also be amplified from the *Staphylococcus aureus* chromosome. Sequence analysis demonstrated that the similarity of the translated ORFs, as well as the genetic organization of this region, are also conserved in *Streptococcus pyogenes* and *Bacillus subtilis*. This raises the possibility that these previously unidentified genes may be important for division in Gram-positive bacteria.

METHODS

Bacterial strains. *Streptococcus pneumoniae* G54 is a recent clinical isolate of 19F capsular type (Pozzi *et al.*, 1996). Fifteen additional *Streptococcus pneumoniae* clinical isolates were chosen from our laboratory strain collection to study the organization of the *dcw* cluster among different *Streptococcus pneumoniae* strains. *Streptococcus pneumoniae* strain Rx1 (Ravin, 1959) was also included in the study as an example of a well-characterized laboratory strain. *Staphylococcus aureus* ATCC 14154 was used for PCR amplification of the *ftsZ–divIVA* region.

DNA manipulation and sequence determination. *Streptococcus pneumoniae* genomic DNA was isolated from the clinical isolate G54 and purified with CsCl as described by Dillard & Yother (1994). The DNA was mechanically sheared, end-repaired and ligated to M13mp18 (New England Biolabs), by using *Bst*XI cloning technology, to eliminate the background due to vector self-ligation and to avoid multiple inserts (Ausubel *et al.*, 1991). Two libraries, having a mean insert size of 0·8 and 1·2 kb, respectively, were constructed. Over 45000 independent clones were sequenced using a PRISM 377 DNA Sequencer (Applied Biosystems). Samples for sequencing were prepared with the PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and sequences were assembled using a custom-designed program (M. De Francesco & G. Feger, unpublished results).

PCR. A series of specific primers (synthesized at Genset Oligos, Paris, France) were designed on the basis of strain G54 sequence information and used for PCR experiments. PCR products were amplified from *Streptococcus pneumoniae* chromosomal DNA with 1 U *Taq* polymerase (Perkin–Elmer) in a 50 µl reaction mixture in a Hybaid Thermocycler. The following temperature profile was used: 94 °C for 60 s to denature, 53–60 °C for 90 s to anneal primers and 72 °C for 120–150 s for extension. When the size of the expected products was larger than 3·0 kb, the Expand Long Template PCR System (Boehringer Mannheim) was used, according to the manufacturer's instructions. The forward primers sp*murG* (5′ GCGATTTGACCTTGGATAGTTTGG 3′) and sp*divIB* (5′ AGGTCAAGGAATATGATATTGTGG 3′) with the reverse primer sp*pyrF* (5′ TGGACGAATCCCTGGTGTCAG 3′), and the forward primers sp*pbp2b* (5′ ACCTTACAAATGGTGTCGGACCT 3′) and sp*murF* (5′ GCAAGAAGGAGTTTCAGAGGAGC 3′) with the reverse primers sp*murF* (5′ GACGAGCCAATTCAGACAAGAGA 3′) and sp*ftsA* (5′ ATGACTTCACGGTCAGGTGTCAT 3′) were used to confirm the organization of the *dcw* cluster (*dcw2* and *dcw1*, respectively) in different *Streptococcus pneumoniae* strains. The forward primer sa*ftsZ* (5′ CACTGGATTCGGAACAAGCGT 3′) and the reverse primer sa*divIVA* (5′ TCAATTTCAGTGCTTAGTTGCTCCA 3′), based on the sequences with GenBank accession nos U94706 and U41072, respectively, were used to amplify the *ftsZ–divIVA* region from *Staphylococcus aureus* chromosomal DNA. When necessary PCR-derived fragments were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced as described above.

Sequence analysis. *Streptococcus pneumoniae dcw* genes were identified by sequence homology searches against the non-redundant database at NCBI, using BLASTP, BLASTP2 and BLASTX programs (Altschul *et al.*, 1990, 1997; Gish & States, 1993). In parallel, homology searches were done against specialized databases: *Streptococcus pyogenes* Genome BLAST Server (Roe *et al.*, 1998) and the *SubtiList* Web Server at Pasteur Institute (Kunst *et al.*, 1997). When necessary, raw nucleotide sequences were downloaded from the database and translated into ORFs using CLONE4 (Educational Software, Harrisburg, PA). Sequence similarities of the deduced gene

products were searched for as described above. Additional computer analysis was performed using the Genetics Computer Group (GCG) package (Devereux *et al.*, 1984).

RESULTS AND DISCUSSION

Cloning, sequencing and characterization of the *dcw* gene cluster of *Streptococcus pneumoniae*

The *dcw* cluster was cloned as part of a whole-genome sequencing project. Two libraries, having mean insert sizes of 0·8 and 1·2 kb, respectively, were constructed and over 45 000 independent clones were sequenced. The correct contig assembly of the *dcw* sequences was verified by PCR. On the basis of strain G54 sequence information, different sets of specific primers were designed and used to amplify the corresponding predicted fragments (varying from 1·5 to 2·0 kb) from G54 chromosomal DNA. In all cases the amplified fragments matched the expected sizes (data not shown).

Three different contigs, containing the equivalent of most of the *dcw* genes, corresponding to the 2 min region of the *Escherichia coli* chromosome were identified. The genetic organization and some features of the identified genes are shown in Table 1. A comparison of *Streptococcus pneumoniae dcw* clusters with the clusters of other bacteria reported to date is shown in Fig. 1. The first region of 10 kb (*dcw1* cluster) contained five *dcw* genes. The *ftsA* and *ftsZ* cell division genes were found at one end of this cluster, while at the other end *pbp2b* (Dowson *et al.*, 1989a) was found. Anomalously, the equivalent homologues of the genes usually found in the region downstream of *pbpB* in other Gram-positive bacteria were missing from the cluster. Instead, this region contained *ddl* and *murF*, so far found only in the *dcw* cluster of Gram-negative species (see Fig. 1). Moreover, additional genes not explicitly related to cell division were found in the *dcw1* region. These genes included a *recM* homologue, located downstream of *pbp2b*, and a *Bacillus subtilis mutT* homologue followed by an ORF specific to *Streptococcus pneumoniae*, located downstream of *murF*. It is worth noting that a gene encoding the *Streptococcus pneumoniae* PBP2b homologue followed by *recM* has been recently identified in the closely related *Streptococcus thermophilus* (Stingele & Mollet, 1996). *murD*, *murG* and *divIB*, always found located upstream of *ftsA*, were found in a second region of 8 kb, separate from the first, and named *dcw2*. In *Streptococcus pneumoniae* G54 the region downstream of *divIB* contained *pyrF* and *pyrE*, encoding homologues of enzymes involved in the sixth and fifth step of pyrimidine biosynthesis, respectively, instead of *ftsA*. A third separate region of 6 kb, named *dcw3*, containing *yllC* and *yllD* but not *yllB*, *pbp2x*

Table 1. Genetic organization and characteristics of the *Streptococcus pneumoniae dcw* gene cluster

Gene	End-points (bp)*	Protein size (aa)	Putative ribosome-binding site and translation start†		
dcw1					
pbp2b	75 > 2117	685	**AGAAA**t**GAG**a	ctgatttgt	ATG aga aaa
recM	2128 > 2724	198	**A**tt**A**gaa**AGG**	aacat	ATG ctt tat
ddl	2900 > 3943	347	tag**AA**tt**AGG**	ac**A**aataat	ATG aaa caa
murF	4027 > 5400	457	tt**AAAGGAG**t	a**GA**a	ATG aaa tta
mutT	5387 > 5998	203	--------	----	ATG aaa cca
orf1	6089 > 6793	234	**A**att**A**t**GAGG**	aaga**C**tag	ATG aat tta
ftsA	7012 > 8385	457	gat**A**ga**GAGG**	aagcgatgtaa	ATG gct aga
ftsZ	8402 > 9661	419	**A**at**AA**a**GAGG**	aa**A**aataaatt	ATG aca ttt
dcw2					
murD	2682 > 4034	450	ga**AA**t**GGAG**t	**T**cgagac	ATG aaa gta
murG	4038 > 5096	352	gt**A**gc**GGAG**t	**TA**aaaagaataaa	ATG aaa aaa
divIB	5106 > 6305	399	cat**AAGGA**aa	gtaa	ATG tca aaa
pyrF	6538 > 7239	233	ta**AAAGGAG**a	aacct	ATG cga gaa
pyrE	7273 > 7905	210	ta**AAAGGAG**a	at**A**c**C**	ATG aca ctt
dcw3					
yllC	1134 > 2084	316	g**GA**ttt**G**t**GG**	**TA**aatagataagat	ATG aca aaa
yllD	2096 > 2413	105	**A**agt**A**a**GAGG**	ga**A**aaag	ATG gca gaa
pbp2x	2417 > 4669	750	**AgAA**ta**G**c**GG**	a**G**taagat	ATG aag tgg
			or **AtA**gc**GGAG**t	aagat	
mraY	4671 > 5651	326	ctttA**GGAG**a	ctaat	ATG ttt att
16S rRNA			3′ OH-UCUUUCCUCC	ACUAG	

* > indicates the direction of transcription.

† Bases shown in bold upper case letters represent bases complementary to 16S rRNA. Dashed lines indicate that no putative ribosome-binding site was found upstream of the identified translational start. Upper case letters in plain type indicate the putative translational start codon.

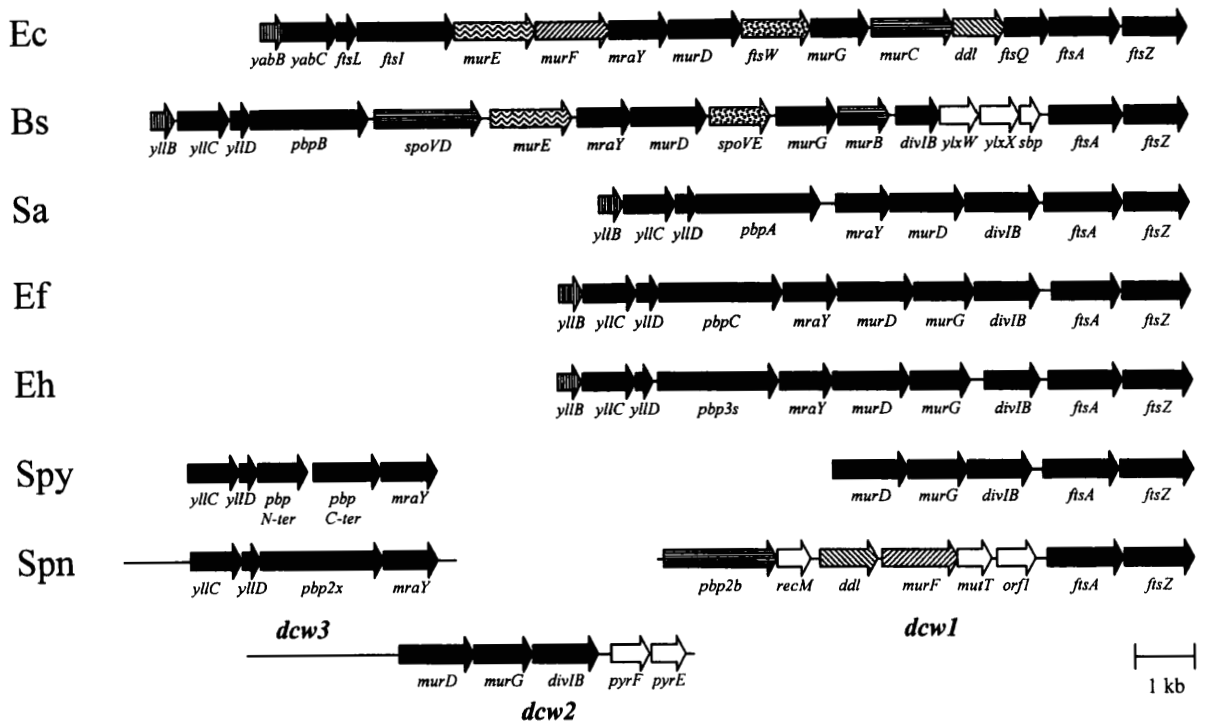

Fig. 1. Comparison of the *dcw* gene clusters of *Escherichia coli* (Ec), *Bacillus subtilis* (Bs), *Staphylococcus aureus* (Sa), *Enterococcus faecalis* (Ef), *Enterococcus hirae* (Eh), *Streptococcus pyogenes* (Spy) and *Streptococcus pneumoniae* (Spn). The coding regions and their direction of transcription are indicated by arrows. Gene names are given under the corresponding coding region. *dcw* genes common to all the species, with the exception of *murG* which is absent in *Staphylococcus aureus*, are shown in black. *dcw* genes found in at least two species are shown with a different pattern. Identical patterns in different species represent structural or functional homologues. *dcw* genes found only in one species are shown with horizontal hatching. ORFs that are not related to *dcw* genes are in white. For the purpose of comparison, coding regions constituting the *dcw1*, *2* and *3* clusters are aligned with the corresponding regions in the other species, as the organization of the chromosome is not yet known.

(Laible *et al.*, 1989) and *mraY* homologues, was also identified. This sequence matched, in part, with a *Streptococcus pneumoniae* sequence in the database (GenBank accession no. Z79691).

Table 2 provides a quantitative description of the relationship between *Streptococcus pneumoniae dcw* predicted proteins and their respective homologues for the bacteria included in Fig. 1. *Streptococcus pyogenes* was excluded from this comparison since the sequence data available are still preliminary (Roe *et al.*, 1998) and translation of the ORFs has not always resulted in full length proteins. As expected, most of the *Streptococcus pneumoniae dcw* gene products, with the exception of YllD/FtsL and DivIB/FtsQ, showed high sequence similarity to their respective homologues, in particular to those of the more closely related enterococci. The highest degree of similarity was scored with the widespread tubulin-like FtsZ, the major component of the septal ring structure (for a comprehensive review see Lutkenhaus & Addinall, 1997). Interestingly, the amino acid sequence corresponding to the GTP-binding motif of *Streptococcus pneumoniae* FtsZ differed from that of all other available FtsZ sequences. This difference is due to a serine residue replacing a threonine in one of the completely conserved positions of the motif (not shown). This substitution may be common to streptococci, since it was also observed in the corresponding GTP-binding motif of the predicted *Streptococcus pyogenes* FtsZ. Studies of the biochemical activities of these proteins (i.e. GTP binding, GTPase activity and polymerization) may help in understanding the biological implication of the observed difference.

Two PBPs, PBP2x and PBP2b (penicillin-sensitive transpeptidases), belonging to the multimodular class B PBPs (Ghuysen, 1991), were found in the *dcw* clusters of *Streptococcus pneumoniae*. Both PBPs account for resistance to β-lactam antibiotics in *Streptococcus pneumoniae* clinical isolates by showing decreased affinity for these drugs (Laible *et al.*, 1991; Laible & Hakenbeck, 1991; Dowson *et al.*, 1989b). At the molecular level, resistance is achieved by replacing entire parts of the *pbp* genes with the corresponding regions from closely related streptococcal species that have PBPs with lower affinities with respect to those of the pneumococcal homologues (Dowson *et al.*, 1989b; Hakenbeck, 1998). One possible explanation for the presence of two PBPs associated with the *dcw* genes is to assure the correct functioning of at least one PBP, since heterologous

Table 2. Comparison of *Streptococcus pneumoniae dcw* gene products

Streptococcus pneumoniae protein	Percentage identity*				
	Escherichia coli	*Bacillus subtilis*	*Staphylococcus aureus*	*Enterococcus faecalis*	*Enterococcus hirae*
PBP2b†	20·7 (569)	32·6 (688)	ND	ND	ND
DdlA	34·3 (251)	43·1 (348)‡	ND	ND	ND
MurF	29·8 (382)	38·0 (458)‡	ND	ND	ND
FtsA	33·0 (358)	40·7 (371)	28·1 (462)	53·2 (370)	57·4 (394)
FtsZ	54·7 (316)	58·0 (374)	52·6 (388)	60·0 (420)	62·9 (428)
MurD	31·3 (450)	49·4 (449)	46·3 (438)	59·6 (453)	58·5 (451)
MurG	27·7 (321)	25·0 (348)	ND	26·4 (364)	24·2 (359)
DivIB§	17·1 (246)	25·3 (265)	20·4 (402)	22·7 (343)	24·4 (271)
YllC‖	40·1 (319)	61·6 (310)	59·4 (315)	66·3 (315)	67·4 (316)
YllD¶	–	22·5 (80)	16·3 (86)	30·0 (100)	29·6 (54)
PBP2x**	24·1 (323)	33·6 (351)	29·8 (429)	37·5 (750)	41·5 (662)
MraY	47·2 (195)	43·1 (318)	42·9 (310)	52·6 (327)	54·8 (310)

* Percentage identical amino acids determined by Lipman–Pearson protein alignment. Numbers in parentheses indicate the length of the amino acid overlap, including gaps. –, No significant similarity found; ND, not determined.

† PBP2 in *Escherichia coli*, PBP2A in *Bacillus subtilis*. The genes encoding these proteins are not located in the *dcw* cluster.

‡ The genes encoding DdlA and MurF in *Bacillus subtilis* are not located in the *dcw* cluster.

§ FtsQ in *Escherichia coli*.

‖ YabC in *Escherichia coli*.

¶ FtsL in *Escherichia coli*.

** PBP3 in *Escherichia coli*, PBP2B in *Bacillus subtilis*, PBP1 in *Staphylococcus aureus*, PBP3 in *Enterococcus faecalis*, PBP3s in *Enterococcus hirae*.

recombinations often occur within the genes encoding these proteins (Dowson *et al.*, 1989b; Hakenbeck, 1998). However, the previously reported findings that both PBP2x and PBP2b are essential (Kell *et al.*, 1993) do not support this hypothesis. Alternatively, it is possible that one PBP is needed for cell wall elongation (PBP2b) and the other for septum formation (PBP2x). This is supported by the fact that PBP2x shows a higher degree of similarity to PBPs associated with septum formation (i.e. *Escherichia coli* PBP3 and homologous PBPs from other bacteria, always located in the *dcw* cluster, see Table 2), while PBP2b shows higher similarity to PBPs involved in lateral elongation (i.e. *Escherichia coli* PBP2 and *Bacillus subtilis* PBP2A), which has been shown to occur in some Gram-positive cocci (Lleo *et al.*, 1990).

Analysis of the *dcw* cluster in different *Streptococcus pneumoniae* clinical isolates

Contig assembly data obtained so far for *Streptococcus pneumoniae* G54 indicates that the *dcw* clusters are physically separated in the chromosome by at least 40 kb each side. This is also supported by experiments with long-range PCR, to connect each cluster, which always resulted in absence of products (data not shown). According to the physical map for *Streptococcus pneumoniae* R6, a distance of at least 385 kb should separate *pbp2x* and *pbp2b* (Gasc *et al.*, 1991).

Recently reported preliminary results indicated that *Streptococcus pneumoniae* has a *dcw* cluster similar to that of other Gram-positive bacteria, in particular *Streptococcus pyogenes* (Pucci *et al.*, 1997). Both streptococci showed the presence of *ftsA* and *ftsZ* at the distal end of the cluster and a PBP-encoding gene, *pbp2x* in *Streptococcus pneumoniae*, followed by *mraY* at the proximal end (Pucci *et al.*, 1997). These findings are different from the results obtained for *Streptococcus pneumoniae* G54 shown in Fig. 1.

To test the possibility that the observed differences reflected strain variability due to genomic rearrangements, the organization of the *dcw* cluster was analysed in 15 additional independent *Streptococcus pneumoniae* strains, including Rx1. Different sets of primers were designed to amplify the region between *murG–divIB* and *pyrF*, *pbp2b* and *murF*, and *murF* and *ftsA*. The sizes of the expected products were calculated on the basis of strain G54 sequence information. The resulting PCR products of two different strains, *Streptococcus pneumoniae* G54 and Rx1, were sequenced (data not shown).

The forward primers sp*murG* and sp*divIB* with the reverse primer sp*pyrF* were expected to give 2123 and 1315 bp fragments, respectively. Of the 15 strains tested, 13 were identical to G54 and products of the expected size were amplified from all of them. In contrast, no amplicons were detected in strain Rx1 and in one other

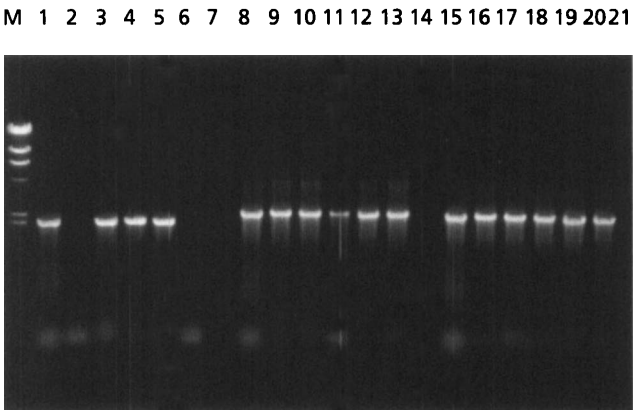

Fig. 2. PCR analysis of *dcw* clusters in different *Streptococcus pneumoniae* strains. Lanes: 1–6, PCR products from strains G54, Rx1, sp186, sp723, sp4636, sp4657 and sp3157, respectively, amplified with primers spF*murG* and spR*pyrF*; 7, negative control (no DNA); 8–13, products from strains G54, Rx1, sp186, sp723, sp4636, sp4657 and sp3157, respectively, amplified with primers spF*pbp2b* and spR*murF*; 14, negative control (no DNA); 15–20 are products from strains G54, Rx1, sp186, sp723, sp4636, sp4657 and sp3157, respectively, amplified with primers spF*murF* and spR*ftsA*; 21, negative control (no DNA); M, *Hin*dIII-digested λ phage DNA.

Streptococcus pneumoniae clinical isolate, even when parameters for long-range PCR were used. These results indicate that *pyrF–pyrE* may not always be located downstream of *divIB* and suggest that chromosomal rearrangements may have occurred outside the *dcw2* cluster in different *Streptococcus pneumoniae* strains. The forward primers spF*pbp2b* and spF*murF* with the reverse primers spR*murF* and spR*ftsA* were expected to give 2512 and 2505 bp fragments, respectively. All the 15 strains tested gave fragments of the expected size, consistent with the results obtained for *Streptococcus pneumoniae* G54. The results for six of these strains are shown in Fig. 2.

Taken together these results indicate that, although some variations were observed among different *Streptococcus pneumoniae* isolates, the genetic organization of the *dcw* clusters of G54 is representative of *Streptococcus pneumoniae*. This organization substantially differs from that of other eubacterial species for which the *dcw* cluster has been characterized.

Identification and characterization of the chromosomal region downstream of *ftsZ* in *Streptococcus pneumoniae* and *Staphylococcus aureus*

Analysis of the region downstream of *ftsZ* in *Streptococcus pneumoniae* revealed the presence of five ORFs transcribed in the same direction as the *dcw* genes and tightly packed together (Table 3). The deduced amino acid sequences of the first four ORFs showed a high level

Table 3. Genetic organization and characteristics of the chromosomal region downstream of *ftsZ* in *Streptococcus pneumoniae* and *Staphylococcus aureus*

Gene	End-points (bp)*	Putative ribosome-binding site and translation start†			Similarity index‡	*P* value§
Streptococcus pneumoniae						
ylmE	9666 > 10337	----------	-----	ATG aat gta	42 (I), 63 (S) (220)	$1{\cdot}3 \times 10^{-46}$
ylmF	10347 > 10886	ttt**AAG**t**AGG**	a**GA**gaacc	ATG tct tta	27 (I), 52 (S) (159)	$4{\cdot}0 \times 10^{-13}$
		or **A**agt**AGGAG**a	ga**A**c**C**			
ylmG	10886 > 11149	gaa**A**ta**GAG**t	acgata	ATG att ttt	36 (I), 60 (S) (75)	$1{\cdot}4 \times 10^{-10}$
ylmH	11146 > 11931	tttct**GG**c**G**a	**TGAT**agg	ATG aat aaa	32 (I), 58 (S) (252)	$3{\cdot}5 \times 10^{-39}$
divIVA	11940 > 12728	gt**AA**gt**GAGG**	aataga	ATG cca att	36 (I), 66 (S) (163)	$1{\cdot}4 \times 10^{-28}$
16S rRNA		OH-UCUUUCCUCC	ACUAG			
Staphylococcus aureus						
ylmD	437 > 1228	**A**tg**A**t**GG**t**G**a	c**GATC**ga	GTG aat gat	31 (I), 55 (S) (247)	$1{\cdot}3 \times 10^{-39}$
ylmE	1246 > 1920	**A**ac**AAGGAGG**	a**GAT**atgt	TTG cgt gtg	55 (I), 72 (S) (224)	$9{\cdot}3 \times 10^{-62}$
ylmF	1926 > 2480	g**GA**g**A**a**GAGG**	a**G**tgagccac	TTG gct tta	52 (I), 67 (S) (129)	$1{\cdot}2 \times 10^{-31}$
ylmG	2492 > 2782	**A**ct**AAGGAG**t	**TA**cat	ATG gat ata	51 (I), 67 (S) (87)	$1{\cdot}7 \times 10^{-21}$
ylmH	2865 > 3671	tat**AAGG**gca	at**A**g	ATG gta ttt	40 (I), 62 (S) (251)	$7{\cdot}1 \times 10^{-48}$
divIVA	3695 > 4312	ttt**AAGGAGG**	a**TA**a**C**aa	ATG cct ttt	41 (I), 72 (S) (158)	$8{\cdot}5 \times 10^{-36}$
16S rRNA		OH-UCUUUCCUCC	ACUAG			

* > indicates the direction of transcription.

† Bases shown in bold upper case letters represent bases complementary to 16S rRNA. Dashed lines indicate that no putative ribosome-binding site was found upstream of the identified translational starts. Upper case letters in plain type indicate the putative translational start codon.

‡ Percentage identity (I) and percentage similarity (S) as determined with the BLAST2 program. Numbers in parentheses indicate the length of the amino acid overlap, including gaps.

§ The *P* value indicates the statistical significance of the alignments. The lower the *P* value, the greater the significance.

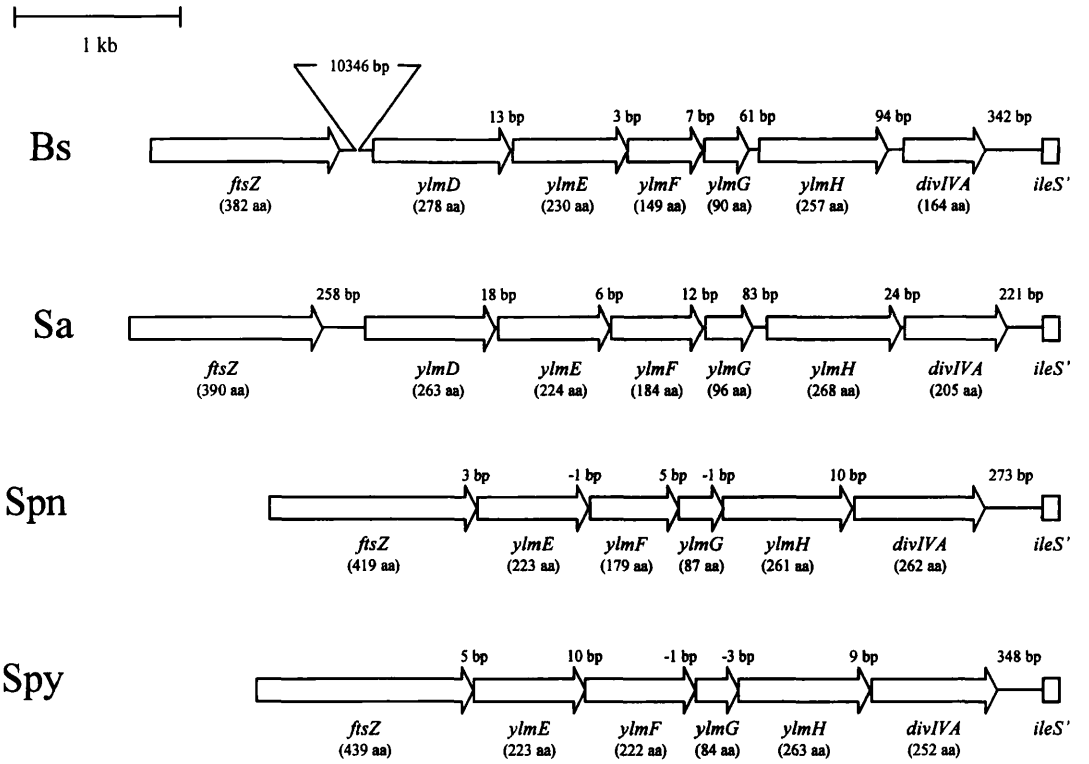

Fig. 3. Comparison of the region downstream of *ftsZ* in *Bacillus subtilis* (Bs), *Staphylococcus aureus* (Sa), *Streptococcus pneumoniae* (Spn) and *Streptococcus pyogenes* (Spy). The coding regions and their direction of transcription are indicated by arrows. Gene names are given under the corresponding coding region. Numbers in parentheses indicate the size of the translated gene products. The gaps between adjacent coding regions are indicated. Note that *ileS* is always located downstream of *divIVA*.

of sequence similarity with the *ylmE*, *ylmF*, *ylmG* and *ylmH* gene products of *Bacillus subtilis* whose functions are unknown. The last ORF showed a high level of sequence similarity (36% identity, 65% similarity over 163 aa) to the *divIVA* gene product of *Bacillus subtilis*, recently found to be involved in division (Cha & Stewart, 1997; Edwards & Errington, 1997), and 31% identity and 56% similarity over 166 aa to a 23·5 kDa protein encoded by an ORF of unknown function located upstream of *ileS* of *Staphylococcus aureus* (Chalker *et al.*, 1994; Grundy *et al.*, 1997). DivIVA also shared sequence similarity with antigen 84 (Hermans *et al.*, 1995; Philipp *et al.*, 1996), a cytoplasmic protein of unknown function of *Mycobacterium tuberculosis* and *Mycobacterium leprae* (22% identity, 40% similarity over 206 aa and 20% identity, 40% similarity over 255 aa, respectively) and the *Bacillus subtilis ypsB* product (38% identity, 64% similarity over 57 aa). *ypsB*, which encodes a hypothetical protein of 98 aa, is located in a region of the *Bacillus subtilis* chromosome that contains the gene encoding PBP1A. Orthologues of this gene are found in a similar position in the *Streptococcus pneumoniae* and *Streptococcus pyogenes* genome (not shown).

From the analysis of the available sequence data we observed that the organization of the genes in the region downstream of *ftsZ* is remarkably conserved in *Bacillus subtilis* and *Streptococcus pyogenes*. To test the possibility that a similar region was also conserved in other Gram-positive bacteria, we designed a forward primer based on the *Staphylococcus aureus ftsZ* gene (Pucci *et al.*, 1997) and a reverse primer based on the ORF encoding the *Staphylococcus aureus* DivIVA homologue (Chalker *et al.*, 1994), taking advantage of the available sequences in the database. These primers were used in PCR experiments to amplify a putative *ftsZ–divIVA* region and to confirm the hypothesis that this region was conserved in other Gram-positive species. Indeed, a 3·7 kb fragment was amplified from *Staphylococcus aureus* chromosomal DNA. The nucleotide sequence of the amplified DNA fragment was determined by primer walking and analysed with CLONE4. The genetic organization and some features of the proteins encoded by the genes located downstream of *ftsZ* and their similarity to the respective *Bacillus subtilis* homologues are shown in Table 3. Fig. 3 shows the map of this region in *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Streptococcus pyogenes* in comparison with that of *Bacillus subtilis*, as taken from the *Bacillus subtilis* genomic database. In *Streptococcus pyogenes* these ORFs are located immediately after *ftsZ* and appear to be part of the same cluster, similar to *Streptococcus pneumoniae*. In *Bacillus subtilis* they also appear to be

clustered together, but lie in a chromosomal region about 10 kb downstream of *ftsZ* (Kunst *et al.*, 1997). As shown in Fig. 3, in *Staphylococcus aureus* and *Bacillus subtilis* an additional gene, *ylmD*, was found upstream of *ylmE*. The deduced amino acid sequences of the *ylmD* genes displayed similarity to a number of proteins of comparable size from other Gram-positive and Gram-negative bacteria. Consistent with the results shown in Fig. 3, all the YlmD homologues of other Gram-positive bacteria (*Mycobacterium tuberculosis*, *Corynebacterium glutamicum*, *Streptomyces griseus*), detected by searching the database, were located immediately downstream of *ftsZ*. However, *ylmD* was not found in streptococci, neither in the region 3′ to *ftsZ* nor elsewhere in the available genome (94% of *Streptococcus pneumoniae* and 95% of *Streptococcus pyogenes*). The *ylmE* gene product showed sequence similarity to a number of hypothetical proteins of comparable size belonging to the UPF0001 family, homologues to the *Escherichia coli yggS* gene product and present in both prokaryotes and eukaryotes (*Saccharomyces cerevisiae* and *Caenorhabditis elegans*). Proteins significantly similar to *ylmF* products were found only in *Mycobacterium tuberculosis* (Philipp *et al.*, 1996) and *Synechocystis* sp. (Kaneko *et al.*, 1996a, b) while significant scores for the *ylmH* products were only detected in *Synechocystis* sp. (Kaneko *et al.*, 1996a, b). Products of the *ylmG* genes of *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Staphylococcus aureus* and *Bacillus subtilis* all shared a good degree of mutual similarity. However, ambiguous results were obtained when individual proteins were tested against the database. In particular, while the streptococcal *ylmG* products gave a significant score only with a similar sized protein of *Synechocystis* sp., the *ylmG* products of *Staphylococcus aureus* and *Bacillus subtilis* shared similarity with proteins of the same or larger size encoded by genes belonging to the *Escherichia coli yggT* family present in several Gram-positive and Gram-negative species. Common to all *ylmG* products was a putative transmembrane domain, in agreement with the prediction that some of the possible distant homologues are integral membrane proteins.

General features of the *Streptococcus pneumoniae dcw* cluster and conclusions

The *dcw* cluster is one of the best examples of highly conserved regions that survived the dynamic rearrangements often observed in eubacterial genomes during evolution (Watanabe *et al.*, 1997). The biological reason for this conservation may be mainly attributed to the fact that this organization facilitates coordinated regulation of gene expression. A complex pattern of regulation within the *dcw* genes has been well documented for *Escherichia coli* (Sullivan & Donachie, 1984; Ayala *et al.*, 1994; Palacios *et al.*, 1996; Cam *et al.*, 1996; Flardh *et al.*, 1997; Hara *et al.*, 1997). It remains to be verified if this can be extended to other eubacteria.

As observed by Pucci *et al.* (1997), in Gram-positive bacteria, unlike *Escherichia coli*, the constituent genes of the *dcw* cluster, perhaps with the exception of *ftsA* and *ftsZ*, are not necessarily tightly packed together and often show intergenic spacing of variable size. This is particularly evident in *Streptococcus pneumoniae* where some of the *dcw* genes are located in physically distinct chromosomal regions. The significance of the unconventional organization observed for the *Streptococcus pneumoniae* cluster is not yet clear, but will possibly emerge from the analysis of the *dcw* clusters of other closely related streptococcal species.

We have also reported the sequence of the chromosomal region downstream of *ftsZ*, consisting of genes transcribed in the same direction as the *dcw* genes. This region appears to be very well conserved in a number of different Gram-positive species (Fig. 3). The last gene of the cluster encodes a protein sharing high sequence similarity with the *divIVA* gene product of *Bacillus subtilis* (Cha & Stewart, 1997). Recently, DivIVA has been proposed to be the *Bacillus subtilis* counterpart of *Escherichia coli* MinE, whose structural homologue is missing in *Bacillus subtilis* and has been shown to act as a pilot protein in determining division-site selection (Cha & Stewart, 1997; Edwards & Errington, 1997). Preliminary results, obtained in our laboratory by disrupting the gene encoding the *Streptococcus pneumoniae* DivIVA homologue, are consistent with a similar role for this protein. Inactivation of *divIVA* in *Streptococcus pneumoniae* resulted in severe growth inhibition and formation of chains of unseparated cells, some of them devoid of nucleoids (O. Massidda, unpublished results). The functions of the genes between *ftsZ* and *divIVA* remain to be evaluated. However, their organization and chromosomal position suggest a possible role in division. Gene inactivation experiments to study the function of the ORFs located downstream of *ftsZ* are currently in progress in *Streptococcus pneumoniae*.

Although the *dcw* cluster has been characterized in many different bacteria, the identification of this cluster in *Streptococcus pneumoniae* provides additional information for the understanding of the evolutionary origin of these genes and their regulation. Moreover, it provides a basis to evaluate, at the molecular level, how division occurs in Gram-positive cocci. Among Gram-positive cocci, streptococci represent a simplified model of division, since they divide only in one plane of space. Furthermore, the ease of genetic manipulation of *Streptococcus pneumoniae* offers a good complement to *Bacillus subtilis*.

ACKNOWLEDGEMENTS

The authors thank E. Domenici for constant advice and support, and M. B. Whalen for helpful discussions. We are grateful to F. Faggioni and M. Guerrini for technical assistance. We also thank J. Coyette and C. Duez for providing us with unpublished results. O. Massidda was sponsored by the Cell Factory Contract No. CT96-0122 from the European Commission (DGXII) Biotechnology Program.

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Received 4 June 1998; revised 14 July 1998; accepted 3 August 1998.