A protein-based phylogenetic tree for Gram-positive bacteria derived from *hrcA*, a unique heat-shock regulatory gene

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The *dnaK* operon from *Bacillus subtilis* and other Gram-positive bacteria with low G+C DNA content contains additional heat-shock genes, including *hrcA*. The *hrcA* gene encodes a transcription factor that negatively regulates heat-shock genes and is uniformly present in all Gram-positive bacteria studied to date. An *hrcA* homologue is also present in *Synechocystis* species, *Leptospira interrogans*, *Chlamydia trachomatis*, *Caulobacter crescentus* and *Methanococcus jannaschii*, organisms that diverged early on from the common ancestor of all Gram-positive bacteria and *Proteobacteria*, according to 16S rRNA phylogeny. A partial, protein-based phylogenetic tree, derived using amino acid sequence homology of *hrcA* proteins from Gram-positive bacteria, is presented here, and the results are compared with the phylogenetic trees generated from 16S rRNA, *dnaK* and *dnaJ* sequences. The location of the *hrcA* gene and the genome organization of the *dnaK* operon support the division of all Gram-positive bacteria into three major groups: one group contains high-G+C Gram-positive bacteria, and two others contain low-G+C Gram-positive bacteria. Among the Gram-positive bacteria with low G+C DNA content, the results indicate that there is a close phylogenetic relationship between *Bacillus* species and *Clostridium* species on the one hand and between *Lactococcus lactis* and *Streptococcus mutans* on the other. *Streptomyces* and *Mycobacterium* species also exhibited a close relationship. A hierarchical arrangement of Gram-positive bacteria based on *HrcA* sequences is proposed as an additional refinement of the phylogenetic relationships within this important bacterial group.

Keywords: *hrcA*, *dnaK* operon, Gram-positive bacteria, phylogeny

INTRODUCTION

The evolutionary relationships among micro-organisms are being determined by partial or complete sequencing of 5S and 16S rRNA genes (Olsen & Woese, 1993; Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 1997; Woese, 1994) mainly because their resistance to evolutionary change allows the entire phylogenetic span of ancient and modern prokaryotes to be analysed simultaneously. However, it has been shown that the resolution power of rRNA sequences is limited when closely related organisms that diverged at almost the same time are being

examined (Fox *et al.*, 1992; Woese, 1987). More recently, sequencing data for other highly conserved molecules (e.g. heat-shock proteins, HSPs) have been used because of their ubiquity and high degree of sequence conservation. Thus, phylogenetic analyses based on Hsp40 (DnaJ), Hsp60 (GroEL), Hsp70 (DnaK) and Hsp90 family of protein sequences have been carried out recently (Boorstein *et al.*, 1994; Bustard & Gupta, 1997; Falah & Gupta, 1994; Gupta, 1995; Gupta & Golding, 1993; Gupta *et al.*, 1989). These analyses have revealed a number of important differences relative to rRNA-based phylogenies (Boorstein *et al.*, 1994; Brown *et al.*, 1994; Gupta & Golding, 1993). However, highly conserved molecules (e.g. 16S rRNA, Hsp70) may not be suitable for determining evolutionary relationships among bac-

Abbreviation: HSP, heat-shock protein.

Fig. 1. Comparison of phylogenetic relationships among Gram-positive bacteria deduced from (a) 16S rRNA sequences (Olsen & Woese, 1993; Stackebrandt *et al.*, 1997; Woese, 1987), (b) the amino acid sequence homology of DnaK proteins (Falah & Gupta, 1997; Gupta & Golding, 1993) and (c) DnaJ protein sequences (Bustard & Gupta, 1997). The trees show the branching order rather than the actual distances. (a) The genera representing Gram-positive bacteria with low-G+C DNA content include, among others, *Bacillus* species, *Clostridium* species, *Staphylococcus* species, *Lactococcus* species, *Streptococcus* species and *Mycoplasma* species.

terial groups that diverged at almost the same time; amino acid sequence homologies, for other proteins that are not so highly conserved, and gene-fusion events may be more appropriate methods (Ahmad & Jensen, 1989; Jensen & Ahmad, 1990). Furthermore, a study based on a very small number of genes could give misleading results if one of the genes had been horizontally transferred across bacterial groups at an earlier stage of divergence. Thus, greater resolution of evolutionary branching can be achieved only by using a greater number of evolutionary trees.

The Gram-positive group of bacteria is of special microbiological interest because of pathogenic and non-pathogenic relationships with mammalian systems, as well as because of its potential for industrial

exploitation. The Gram-positive bacteria have evolved only recently from a common ancestor (Woese, 1987). Their recent evolutionary origin means that highly conserved molecules, such as 16S rRNA sequences, could not resolve the branching order, as the divergence among various groups occurred at almost the same time (Rainey *et al.*, 1995; Stackebrandt *et al.*, 1997; Woese, 1987). Thus, comparative sequence analyses of other cistrons that are less highly conserved may help to clarify the hierarchical order.

A comparison of the phylogenetic trees constructed by means of 16S rRNA sequencing (Stackebrandt *et al.*, 1997; Woese, 1987) and amino acid sequence homology of DnaK (Falah & Gupta, 1994; Gupta & Golding, 1993) and DnaJ (Bustard & Gupta, 1997) proteins for several Gram-positive bacteria is shown in Fig. 1. Almost the same set of organisms are used. The resolution and the discrepancies in the branching order in the three trees are clear. The order of branching among Gram-positive bacteria with low G+C DNA content could not be resolved on the basis of 16S rRNA sequences. The tree based on Hsp70 sequences depicts *Clostridium* species branching off early before the divergence of *Mycoplasma*/*Lactococcus*/*Staphylococcus* species from the *Bacillus* species. Both *Bacillus* species and *Clostridium* species sporulate (Errington, 1993), a process that involves the expression of several genes. The tree based on DnaJ sequences shows *Clostridium* species branching off from the low-G+C DNA organisms (e.g. *Bacillus* species) even earlier than other high-G+C DNA organisms (Bustard & Gupta, 1997). Furthermore, the branching order places *Mycobacterium tuberculosis* closer to *Streptomyces coelicolor* than to *Mycobacterium leprae*, a result that is highly unlikely to be valid (Cole *et al.*, 1998).

The discrepancies in the branching order could be because of horizontal transfer of a gene at an earlier point of divergence or because of different mutation rates for these genes in various organisms following their divergence. The genes encoding HSPs are ubiquitous and essential for cell survival. However, the possibility that horizontal gene transfer is responsible for the varying results obtained with DnaK and DnaJ phylogenies cannot be ruled out in view of the presence of more than one gene copy for *dnaJ* homologues in some Gram-positive bacteria (Grandvalet *et al.*, 1998). Both of the above points can be effectively addressed and the branching order resolved by simply analysing a greater number of carefully chosen cistrons; this is the basis of the study presented here.

The *dnaK* operon from *Bacillus subtilis*, *Bacillus sphaericus* and other Gram-positive bacteria contains two, three or several other heat-shock genes, including *hrcA*, *grpE* and *dnaJ* (Ahmad *et al.*, 1999; Eaton *et al.*, 1993; Falah & Gupta, 1997; Mogk & Schumann, 1997; Narberhaus *et al.*, 1992; Ohta *et al.*, 1994; Tokunaga *et al.*, 1998; Wetzstein *et al.*, 1992). The *hrcA* gene encodes a novel, heat-shock regulatory gene

(Roberts *et al.*, 1996; Schulz & Schumann, 1996) that is distributed throughout Gram-positive bacteria and is an ideal basis for constructing phylogenetic relationships. In this paper, we present a phylogenetic tree for several Gram-positive bacteria, based on the genome location of the *hrcA* gene (relative to the *dnaK* operon) and, for the first time, amino acid sequence homology comparisons for the HrcA protein.

METHODS

The cloning, sequencing and genome organization of the *dnaK* operon from *B. sphaericus* has been described previously (unpublished; EMBL accession no. Y17157). The *dnaK* operon contains at least five protein-coding genes in the order *hrcA*–*grpE*–*dnaK*–*dnaJ*–ORF35 (Ahmad *et al.*, 1999). The *hrcA* gene was translated and a FASTA search of EMBL, GenBank and other databases was performed using the encoded protein sequence (Altschul *et al.*, 1990). The sequences of *hrcA* homologues from various Gram-positive bacteria, identified in FASTA searches, were retrieved. The EMBL accession numbers of the *hrcA* homologous sequences from the database are as follows: *B. subtilis*, P25499; *Bacillus stearothermophilus*, O45550; *Clostridium acetobutylicum*, P30727; *Staphylococcus aureus*, P45556; *Lactococcus lactis*, P42370; *Streptococcus mutans*, O06940; *Mycoplasma pneumoniae*, P75351; *Mycoplasma genitalium*, P47447; *Mycoplasma capricolum*, U51235; *Mycobacterium tuberculosis*, O05824; *Mycobacterium leprae*, Q49772; *Streptomyces albus*, AF025656; *Leptospira interrogans*, G2735759; *Synechocystis* species, P72795; *Chlamydia trachomatis*, P54306; and *Caulobacter crescentus*, U33324. Various *hrcA*-encoded sequences were initially aligned using the CLUSTAL program (PC/GENE software) (Myers & Miller, 1988) and then inspected for any obvious misalignments. The amino acid sequence identity between pairs of proteins was calculated for the entire length of all HrcA proteins by using the FASTA program (Altschul *et al.*, 1990). The partial sequence from *Mycobacterium leprae* was not used for pairwise comparisons. However, the phylogenetic analyses were carried out with the 195 N-terminal amino acid residues of all *hrcA* homologues corresponding to the partial sequence of HrcA protein that is available for the *Mycobacterium leprae* homologue. A phylogenetic dendrogram was constructed using the treeing algorithms contained in the PHYLIP (version 3.5) phylogenetic software package. The robustness of the tree topologies was evaluated by bootstrap analysis (100 resamplings) of the neighbour-joining data.

RESULTS AND DISCUSSION

The *dnaK* operon from *B. sphaericus* encodes at least five heat-shock proteins arranged in the order *hrcA*–*grpE*–*dnaK*–*dnaJ*–ORF35. The location and genome organization of the *hrcA* gene in relation to the other *dnaK*-related genes (*hrcA*, *grpE*, *dnaK* and *dnaJ*) of the *dnaK* operon were compared using various Gram-positive bacteria and other bacterial groups that possess an *hrcA* homologue. The results are shown in Fig. 2. The organization of these four heat-shock genes is identical in one cluster of Gram-positive bacteria that have low G + C content in their DNA; that cluster included *Bacillus* species, *Clostridium* species, *Staphylococcus* species and *Mycoplasma* species (Ahmad *et*

al., 1999; Falah & Gupta, 1997; Mogk & Schumann, 1997; Narberhaus *et al.*, 1992; Ohta *et al.*, 1994; Tokunaga *et al.*, 1998; Wetzstein *et al.*, 1992). A similar gene arrangement within the *dnaK* operon is also found in the spirochaete *Leptospira interrogans* (Ballard *et al.*, 1998). Although the *hrcA* gene is part of the *dnaK* operon in both *Lactococcus lactis* and *Streptococcus mutans*, the *dnaJ* gene is not encoded by the *dnaK* operon (Eaton *et al.*, 1993; Jayaraman *et al.*, 1997; Koch *et al.*, 1998). The gene order *hrcA*–*grpE*–*dnaK* found within the *dnaK* operon of both *Lactococcus lactis* and *Streptococcus mutans* is unique to these Gram-positive bacteria with low G + C DNA content. The chromosome location of the *hrcA* gene and the organization of the *dnaK* operon, however, differ in Gram-positive bacteria with high G + C DNA content. The *hrcA* gene is present independently, while the *dnaK* operon contains the remaining three genes. Furthermore, the gene order *dnaK*–*grpE*–*dnaJ* within the *dnaK* operon is unique to the high-G + C DNA group of Gram-positive bacteria examined to date (Bucca *et al.*, 1995; Cole *et al.*, 1998; Grandvalet *et al.*, 1998).

Thus, the chromosome location and genome organization of the four heat-shock genes (*hrcA*, *grpE*, *dnaK* and *dnaJ*) allow the categorization of all Gram-positive bacteria into three groups. One group includes high-G + C Gram-positives, whereas low-G + C Gram-positives fall into two groups. The clusters roughly reflect the grouping of these organisms on the basis of 16S rRNA sequencing data (Olsen & Woese, 1993; Stackebrandt *et al.*, 1997; Woese, 1987).

The *hrcA*-encoded protein sequence from *B. sphaericus* was compared with all the protein sequences in various databases using FASTA searches and the homologous sequences were retrieved. Thus, a total of 13 complete HrcA protein sequences, in addition to a partial sequence for *Mycobacterium leprae*, were obtained from various Gram-positive bacteria. These sequences corresponded to both of the major groups (high G + C DNA content and low G + C DNA content) of Gram-positive bacteria. A multiple alignment based on *hrcA*-encoded amino acid sequences from Gram-positive bacteria is shown in Fig. 3. Two highly conserved regions have been identified from all the *hrcA* sequences (labelled in Fig. 3 as Box 1 and Box 2). The N-terminal 80 amino acids of the *hrcA* protein sequences exhibit maximum identity and contain one of the two highly conserved domains (Fig. 3, Box 1). This is similar to the amino acid sequence identities observed with Hsp40 (DnaJ) sequences (Bustard & Gupta, 1997).

The FASTA program and the PALIGN program (PC/GENE software) were used to make a pairwise comparison of the *hrcA*-encoded protein sequences from representative species of Gram-positive bacteria. The amino acid sequence of the HrcA protein from *Synechocystis* species (Glatz *et al.*, 1997) was included because of the reportedly close association between cyanobacteria

Fig. 2. Chromosomal locations of *hrcA* gene homologues and the organization of the *dnaK* operon from diverse Gram-positive bacteria, including *Leptospira interrogans* (Spirochaetales) (Ballard et al., 1998), *Chlamydia trachomatis* (Chlamydiales) (Tan et al., 1996), *Synechococcus* species (cyanobacteria), *Methanosarcina mazei* (Archaea) (Conway de Macario et al., 1994) and *Escherichia coli* (Proteobacteria) (Lipinska et al., 1988; Saito & Uchida, 1978). The chromosome locations of *hrcA* homologues are shown in relation to the other genes encoded by the *dnaK* operon. Although *Leptospira interrogans* and *C. trachomatis* have gene arrangements similar to those exhibited by the first and second groups of low-G+C DNA, Gram-positive bacteria, they are shown separately because the other genes in the vicinity vary.

and Gram-positive bacteria (Bustard & Gupta, 1997; Woese, 1994). This analysis indicated that the amino acid identity between various homologues varied between 24 and 80% over the entire length of these proteins (Table 1). The *hrcA* homologues from various *Bacillus* species exhibited maximum identity to each other and significantly higher homology with the corresponding protein from *Clostridium acetobutylicum*, another spore-forming, Gram-positive bacterium with low G+C DNA content relative to HrcA sequences from other Gram-positive bacteria (Table 1). The amino acid sequence of the *hrcA* homologue from *Mycobacterium leprae* is known only for the N-terminal 195 residues and exhibited 90% identity with the corresponding protein from *Mycobacterium tuberculosis* (data not shown). The two mycobacterial sequences also exhibited higher amino acid identity with the corresponding protein from *Streptomyces albus* (another Gram-positive bacterium with high G+C DNA content) than they did with other sequences. For a large number of sequence pairs, the amino acid identity was in the range 24–32%, which is similar to the overall identity observed for DnaJ protein sequences (Bustard & Gupta, 1997) but lower than that observed for Hsp70 protein sequences (Falah & Gupta, 1994). This suggests that HrcA and DnaJ proteins are structurally not as highly conserved as the Hsp70 protein.

The amino acid sequence alignment of *hrcA* homologues shown in Fig. 3 was used to examine the phylogenetic relationships between various Gram-positive bacteria. For this purpose, only the sequence

of the 195 N-terminal amino acid residues from various *hrcA* homologues was utilized, including the partial sequence from *Mycobacterium leprae*. The phylogenetic relationships between various Gram-positive bacteria were deduced by using the PHYLIP phylogenetic software package. To determine the reliability of the branching order, the sequences were bootstrapped 100 times (Felsenstein, 1985).

A neighbour-joining consensus tree based on HrcA sequences is shown in Fig. 4. The bootstrap scores observed for all the nodes are indicated. In general, the long branch lengths at deeper positions are characterized by low bootstrap scores. No members belonging to the low-G+C DNA group of Gram-positive bacteria share an immediate common ancestor. The three *Bacillus* species, *Clostridium acetobutylicum*, and *Staphylococcus aureus* are clustered together before the branching of the common ancestor of the *Bacillus* species, while *Staphylococcus aureus* branched off at the deepest position within this cluster. Other members belonging to the low-G+C DNA content category failed to cluster within this grouping. *Lactococcus lactis* exhibited a close relationship with *Streptococcus mutans*. However, these bacteria, together with *Mycoplasma* species belonging to the low-G+C DNA group, diverged before the Gram-positive bacteria with high G+C DNA content, i.e. from the main lineage of low G+C group. These clusters are characterized by long branch lengths, perhaps indicating a tentative hierarchical order at deeper positions. It is interesting to note that

		Box 1		58
Basu	M	LTNRQLLILQVIINDFIKSAQPVGSRTLSKKDEITFSSATIRNEMADLEELGFIET		
Bast	V	-E-----D-----R-G-----H--AL-----Y----		
Basp		-----Q-----D-----L-----QI-----P-----T-----YL----		
Cloab		-EMEE-K-K--A--Y-NNGE-----IA--YNLGI-----M-Y--QL		
Staa		-I-D--S--NA-VE-YVDFG-----K--IERHNLNV-P-----KQ--D-NY--		
Mycge		-KN--P--AQ--KA--EY-AY-I--KL-T--YFKNL-GG-L-----A--KK--LK-N		
Mycpn		-KN--T--AQ--KA--EY-AYPV--KL-T--YFKNL-GG-L-----V--KE--YLN-N		
Lacla		-I-E--RQ--NL-VSLYAKDHT-I--KS-LDS IQA-----D-KA--R--L-Q-E		
Strmu		-I-Q--KD--NL-VEL-T-THE-I--K--QNS -AS-R-----D--A--KL--LL--A		
Mytub		-GSADE-RFEV-RA-VA--VATQE-I--KS-VERHNLGV--V--D--V--AE-Y-TQP		
Mylep		-GNADE-RFEV-RA-VA--VATKE-I--A-VERHNLGV--D--V--AE-Y-TQP		
Stmal		-SE-R-EV-RA-VQ-YVGTEE-----KALTERHRLGVSP--V--D--A--DE-Y-AQP		
		Box 2		116
Basu	HSS	SGRVPSKGYRYVVDHLLSPVKLTQSDQIHISFKE KIFELEKTVQKSAQLSD		
Bast	-V-	-----QR--D-IQK-K--A--R-Y--V-----		
Basp		-----F-----QGINSR-IQ--Q-T-ND RLV-V-H-IRR--N--E		
Cloab	-T-	-K--D--L--R-MEIPSMSEEMML-KAKIIDSALY-ID-L-KQAMSLV-E		
Staa		-S--QL-F--NR--EQTSQ--TNKLRRLNQLLVENQYDVSSALTYF-DE-N		
Mycge	-I-	-----QI--Q--KV-NVSNITN DLKTRLSVILQ QHKTIDEVIELGVKFINE		
Mycpn	-I-	-----QL--Q--KL-TKNDKKS NLKTRLRRA-ILQ KHKTIIDEIELGVKFINE		
Lacla	-T-	-I--VS--K-F-ENVIOLEEFSSQNDLFKYMKA-DG DFYR-SDLFKTA-KS-E		
Strmu	TPPAVVC-VK-AT-	-F-EHS-N-DS-DEQ-VY-VIKA-DF EAFR-GDLL-R-SDV-AN		
Mytub	-T-	-----EF--R-EDVKP-S- AERRAIQS-L- SGVDLDDVLRRAVRL-AC		
Mylep	-T-	-----EF--R-EDVKP-S- AERRAIQS-L- SGVDLDDVLRRAVRL-AC		
Stmal	-T-	A-PI-TD--LF--R-ADV KPM- APERRAIHH-LD QAQV-DDVARTVRL-AH		
		Box 2		176
Basu	LTNYTSIVLGPKNSENYLKQIQIPIQPDMAVILVNTNGHVENKTINFPTKMDLSDEIK			
Bast	-A--AVK--K-R--LNQQT--I--D-----HV-TV-ASVNP--L--			
Basp	-S-----L--DVQRHVR-RFS-V-LSS-T--I--N-----RLF-L-PDFTA--L--			
Cloab	M-KL-CV-KSLARKS-I-S-SL-N-E-NMILCVFI-DS-MTK-SI-RVKSNEN-SL-R			
Staa	ISQ--TL-VH-NHKQDINNVI--RAN-NLVIMVI-FSS--HVHLASDIPFSSNKLNT			
Mycge	II- LPV--TNFS-DEV--K-DL-ILDKSF-LFL--SAS-K-FK--SYANGROFE--VI			
Mycpn	MV- LPV--THFS-DEV--K-DL-MLDQSCALL--SAS-N-FK--SYANGROFE--MV			
Lacla	--GL-F--NAPQRDQ-VSEFMVM-DNHSVLSVITLG--E-RTNQIFLPKSMTEA-AV			
Strmu	--GY-ALI-DVEPKQR--TTFD-VKLSNHD-L-V-TLDEASPTVQFALPKNFELD--LMT			
Mytub	--RQVAV-QY-T--TSTVRHLEV-ALTPARLLMVVI-DS-R-DQRIVELGDVI-DHQLAQ			
Mylep	--CQVAV-QY-TL-TSTVRHLEV-ALS-ARLLMVVI-ES-R-DQRIAEELGDVI-DYQLSQ			
Stmal	V-RQVAV-QY-S-TRSTVRHVELLSMAPARLLMLV-I-D-R--QRM-DC-APFGE-SVAD			
		Box 2		234
Basu	LVNINLDRLSGVPMDLENE RIFKEVVMYRLQHKYKNYLDALRSTFSTHNVKELFF			
Bast	M--F-E--I--LVD-KD K-Y-K-ADVLKR--R--SM-QTIVE-LDIP QE--M--			
Basp	M-----T--SLE--HK SLEA--LAVLQ--VQSA-DFYPR-TNSNATIQKA-I-Y			
Cloab	IA--SK-K-LTIEQINL EVINNIKKDLREYGHIFDC-MPN-YDILREADSTEYKE			
Staa	IS-FVNTN-TFVNQNLQDD I-SFVQSEGEIIFI-K-INTNMHIN QSNISYN			
Mycge	C-R-F--I-IDTRFS-I-ECLEVL--IIRTKVHEYQYVIDEILFKLPDLQIEAN -KIY			
Mycpn	C-R-F--I-IDTRFCDIAQHLDVL--IIRSKVHEYQYVIDEILFKLPDEFQQAQ-QVY			
Lacla	FS-LVKE--V-KKVIDIHY TLRT-IPQIQYRQY-VTSEV-QLFE-I-DDLKEHLTA			
Strmu	VAK-ARE-FLNQTVLDIHY TLRT-IPQIQYRQY-VTSEV-QLFE-I-DDLKEHLTA			
Mytub	-RE GOA-E-KKLSAASV AVADLASQLGGAGGLGDAVGRAATVLLSLEVEHE-R-LL			
Mylep	-REM-SQAMG-KKLSAASAR			
Stmal	-RAR--SQVA-RRFADVPQLVQDLP-SFESED RPTVSTVLSTLLETVEETE-R-MI			
		Box 2		293
Basu	GGKINMLNQPEFHIDITRVSRLSLIEKQDVQLKLVQSP HT GISIKIGKENDYEEMENC			
Bast	A-T-----N--QKI-P-MKM--Q-K-FYR-LRKHNRK --QVT--R--QLS----			
Basp	---T--F-----LNK--MI-D-M-TTSQ-QS-FHNP ES --H-R-S--KQL----			
Cloab	-TMNIFN Y--K--EKAKEF--V-DDRILDT-FNAGS -VTVN--N--SDS-ARDF			
Staa	--VKLIDALNESNVSSIQPI--QY--SNRIAE-LQDISFN -NV-----I-DK LSDI			
Mycge	-IQYLAQ ---ANQEKLTKI-N-L-DTSVWQMAFINTQNTKTN-VF-DQLGFK EI			
Mycpn	-IHYLAQ ---ANQERITRI-N-L-DTSVWQMAFINTQNTKTN-TF-DKLGLE GE EV			
Lacla	-HKNIFDYATDNL AELYK-F-DD-RMLHEIREITNN DE MRAVKFOND EKEMKNL			
Strmu	-KIKTLFAG LDTYQF-ENLQSVALEIRQSLPE DE LHRVQVADSKEKSLADL			
Mytub	--TA-LT RNAADFSGSLI--I-EAL--QVY--R-LAAQEQAGKVTVR--H-TAS-Q-VGT			
Mylep	--TA-LT RFGHDFPLTI-PV-EAL-EQVVL---LGEAKDS AMTVR--H--AH-GLSST			
Stmal				
		Box 2		343
Basu	SLITASYSVDQKQIGSIAIIGPTRMNYSRVSLQHVTSDLSKALTSLYDE			
Bast	-----T--IGDE-L-T--L-----E-----ITI-NRVA-----I--KN-QNG			
Basp	-V--TT--IGDD-Q-A--D-----D-K--A--DVIRL--TQ-F-KNRS-			
Cloab	-VVSSV-KYNGRPL-T-G-----IP--K--IKVIME-VQIINNND-KMNNNS			
Staa	-IV-SQ-HF-ETLK-Q--V-----A-H-QN-IQ--NRIW			
Mycge	-VASTLINTTSEAKHQL--V-----D-QKIK-A--TLKEEIE-YDKKIHNQT			
Mycpn	-VASTLINTTSEAKHQL--V-----ND-QK-KA--LTLKEEIEEYDKLHGKGTSSST			
Lacla	TI--SQKFVIPPYRGF-TLTVV--VE-D-Q-TL-V-DL-AKV-TMK-SDY-RYLDGNHYEISK			
Strmu	TV--SQKFLIPPYRGF-ILTV--VDLD-Q-TI--INVISRV-AVK-GDF-RYLSNHYEVH			
Mytub	-MVSTA-GTAHTVY--GMGVV-----D-PGTIASVAA--ALYIGDV-GAR			
Mylep				
Stmal	-VVSVG-GSGREAVAKLVV-----D-PGTMGAARA-ARYVQGI-AES			

Fig. 3. Multiple alignment of *hrcA*-encoded sequences used in this study, from various Gram-positive bacteria. Abbreviations for species names are as follows: Basu, *Bacillus subtilis*; Bast, *Bacillus stearothermophilus*; Basp, *Bacillus sphaericus*; Cloab, *Clostridium acetobutylicum*; Staa, *Staphylococcus aureus*; Lacla, *Lactococcus lactis*; Mycge, *Mycoplasma genitalium*; Mycpn, *Mycoplasma pneumoniae*; Strmu, *Streptococcus mutans*; Mytub, *Mycobacterium tuberculosis*; Mylep, *Mycobacterium leprae*; and Stmal, *Streptomyces albus*. Amino acids

Lactococcus lactis and *Streptococcus mutans* cluster together and are not closely related to the *Bacillus* species. This is also shown by the organization of the *dnaK* operon, which contains the *hrcA*-*grpE*-*dnaK* genes but not the *dnaJ* gene (Fig. 2). *Mycobacterium tuberculosis*, *Mycobacterium leprae* and *Streptomyces albus*, all of which are Gram-positive bacteria with high G+C DNA content, formed another cohesive group. The positioning of *Synechocystis* species on the basis of the *hrcA* sequence suggests that the Gram-positive bacteria do not form a monophyletic group. The positioning of *Synechocystis* species may be a consequence of the long branch effect (Felsenstein, 1985). Phylogenetic analyses carried out with all of the *HrcA* sequences resulted in a similar tree, except, of course, that *Mycobacterium leprae* was not represented (data not shown).

The tree exhibited that the three *Bacillus* species used in this study and *Clostridium acetobutylicum* share a common ancestor. This hierarchical arrangement demonstrates a close evolutionary relationship among these bacteria, which also share sporulation, a complex characteristic that involves the expression of several sporulation-dependent genes (Errington, 1993). The phylogenetic analyses derived from 16S rRNA gene sequences have not resolved the branching order within low-G+C DNA, Gram-positive bacteria (Olsen & Woese, 1993; Stackebrandt *et al.*, 1997; Woese, 1987). However, phylogenetic trees derived from amino acid sequence homologies of two proteins encoded by the *dnaK* operon (*DnaK* and *DnaJ*), as well as from Hsp60 sequences, place these organisms in separate clusters (Boorstein *et al.*, 1994; Bustard & Gupta, 1997; Gupta & Golding, 1993) (see also Fig. 1).

The discrepancies in the branching orders noted above could be the result of several factors that may operate independently. It is known that some genes can tolerate fairly high mutation rates without adverse effects on their functioning. Consequently, mutation rates for the gene under study could vary dramatically in different organisms following their divergence. Thus, *dnaJ* and *hrcA* homologues exhibit a minimum overall identity of 25%, whereas the minimum identity exhibited by any two *dnaK* homologues is almost 45% (Bustard & Gupta, 1997; Falah & Gupta, 1994). It is also possible that the gene under study was acquired through horizontal gene transfer in one lineage at an earlier stage of divergence. It has been reported that some bacterial genera including Gram-positive bacteria contain more than one copy of *dnaK* and *dnaJ* homologues (Grandvalet *et al.*, 1998; Nimura *et al.*,

are shown according to the standard one-letter code, and residues identical to those of *B. subtilis* are indicated by dashes. Gaps, indicated by blank spaces, are introduced in order to obtain maximum fit. The two highly conserved domains are labelled 'Box 1' and 'Box 2'. Asterisks highlight the insertions or deletions that could serve as 'signatures' in various sequences.

Table 1. Similarity matrix of *hrcA* sequences

The pairwise amino acid identity (%) for the entire length of various *hrcA* homologues was calculated by using the FASTA program and the PALIGN program from PC/GENE software.

	1	2	3	4	5	6	7	8	9	10	11	12
1. <i>Bacillus subtilis</i>	100											
2. <i>Bacillus stearothermophilus</i>	64	100										
3. <i>Bacillus sphaericus</i>	56	56	100									
4. <i>Clostridium acetobutylicum</i>	35	35	33	100								
5. <i>Staphylococcus aureus</i>	32	32	30	29	100							
6. <i>Mycoplasma genitalium</i>	31	29	30	27	27	100						
7. <i>Mycoplasma pneumoniae</i>	32	29	29	29	26	80	100					
8. <i>Lactococcus lactis</i>	27	28	26	26	25	26	26	100				
9. <i>Streptococcus mutans</i>	27	26	28	22	25	22	24	45	100			
10. <i>Mycobacterium tuberculosis</i>	29	29	29	31	27	23	24	26	24	100		
11. <i>Streptomyces albus</i>	27	28	27	30	28	25	27	24	24	56	100	
12. <i>Synechocystis</i> species	30	29	29	24	24	26	27	24	24	27	27	100

Fig. 4. Consensus, bootstrap, neighbour-joining, phylogenetic tree for Gram-positive bacteria based on *HrcA* sequences. The numbers on the nodes indicate the number of times (%) the species (shown on the right) grouped together in 100 bootstrap samples. The phylogenetic analysis was carried out on the alignment of 195 N-terminal amino acid residues of various *HrcA* sequences for which sequence information is available (including *Mycobacterium leprae*, as shown in Fig. 3).

1994; Ward-Rainey *et al.*, 1997; Yoshimune *et al.*, 1998). The extent of this duplication across various genera is not known at present. It is also unclear whether multiple copies originated in the same organism through gene duplication or were acquired by

other mechanisms. It is probable that one of the gene copies was acquired through horizontal transfer in these genera. If so, this may explain some of the varying results obtained with *hrcA*, *dnaJ* and *dnaK* phylogenies.

The overall homology of HrcA proteins roughly resembles that exhibited by DnaJ sequences (Bustard & Gupta, 1997) and provides an alternative hierarchical order among Gram-positive bacteria. The availability of a greater number of phylogenetic trees based on a large number of conserved and ubiquitous cistrons is expected to allow the establishment of evolutionary relationships and hierarchical order among important bacterial groups, such as the Gram-positive bacteria.

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