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Carbamoyl-phosphate synthetases (CPS) in lactic acid bacteria and other Gram-positive bacteria

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Abstract — Carbamoyl-phosphate synthetases (CPS) catalyze carbamoyl phosphate (CP) biosynthesis from glutamine, bicarbonate and ATP. CPS are formed of two subunits, a small glutaminase subunit and a large synthetase subunit. CP is a common intermediate of arginine and pyrimidine biosynthesis. CPS in prokaryotes are either arginine-regulated (CPS-A), pyrimidine-regulated (CPS-P) or regulated by both components. Two to zero CPS are present in the four lactic acid bacteria studied (*Lactobacillus plantarum, Enterococcus faecalis, Lactococcus lactis* and *Lactobacillus delbrueckii* ssp. *lactis*). Only *L. plantarum* harbours two CPS with a CPS-P providing CP for both metabolic pathways. CPS-A can only supplement CP for arginine biosynthesis in higher concentrations of CO_2 or bicarbonate. The CPS-P present in *L. plantarum* and *E. faecalis* is encoded by genes within the *pyr* operon, and genes dispersed within the chromosome in *Lc. lactis*. CPS is absent in *L. delbrueckii* ssp. *lactis* and the catabolism of arginine via the arginine deiminase pathway (ADI) provides the CP for pyrimidine biosynthesis. In addition to their functional CPS-P, *E. faecalis* and *Lc. lactis* also harbour an ADI pathway so that arginine catabolism may regulate CP biosynthesis in these species. Lactic acid bacteria CPS were compared to CPS of 13 Gram-positive bacteria with sequenced or partially sequenced genomes. Most organisms harbour a CPS-P. CPS-P is also found in the few organisms (*L. plantarum, B. subtilis* and *B. stearothermophilus*) which harbour a CPS-A. The number of CPS and the organization of their genes is variable in Gram-positive bacteria.

arginine / pyrimidine biosynthesis / lactic acid bacteria / Gram-positive bacteria / carbamoyl-phosphate synthetase

Résumé — La diversité des carbamyl phosphate synthétases (CPS) des bactéries lactiques. Les carbamyl phosphate synthétases (CPS) sont les enzymes responsables de la biosynthèse du carbamyl phosphate (CP) à partir de glutamine, de bicarbonate et d'ATP. Les CPS sont des enzymes hétérodimériques formées d'une petite sous-unité glutaminase et d'une grande sous-unité synthétase. Le CP est un intermédiaire commun des voies de biosynthèse de l'arginine et des pyrimidines. Chez les procaryotes, il existe des CSP-A réprimées par l'arginine, des CPS-P régulées par les pyrimidines et des CPS régulées par les deux voies de biosynthèse. Dans les 4 bactéries lactiques étudiées (*Lactobacillus plantarum, Enterococcus faecalis, Lactococcus lactis, Lactobacillus delbrueckii* ssp.

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lactis), le nombre de CPS varie de 2 à 0. *L. plantarum*, *Lc. lactis* et *E. faecalis* possèdent une CPS-P régulée par un mécanisme d'atténuation transcriptionnelle pyrimidine-dépendant. Les gènes codant la CPS-P sont dispersés sur le chromosome de *Lc. lactis* et regroupés au sein d'un opéron codant les enzymes de la voie de biosynthèse des pyrimidines chez *L. plantarum* et *E. faecalis*. *L. plantarum* possède également une CPS-A régulée par l'arginine, comme chez *Bacillus subtilis*. Une particularité de la CPS-A de *L. plantarum* est sa dépendance vis-à-vis du CO_2 (ou de sa forme dissoute, le bicarbonate). Cette CPS-A pourrait avoir une faible affinité pour son substrat, le bicarbonate, ou le pool intracellulaire de CO_2/bicarbonate régulerait le métabolisme du CP chez *L. plantarum*. *Lactobacillus delbrueckii* ssp. *lactis* (anciennement *Lactobacillus leichmannii*) ne possède pas de CPS et c'est la dégradation de l'arginine via la voie de l'arginine déiminase (ADI) qui fournit le CP nécessaire à la biosynthèse des pyrimidines. *E. faecalis* et *Lc. lactis* possèdent une CPS-P et une voie de l'ADI et, dans ces deux organismes, le catabolisme de l'arginine pourrait réguler la biosynthèse du CP et donc des pyrimidines. Les CPS des bactéries lactiques ont été comparées aux CPS de procaryotes bien étudiés, ainsi qu'à celles de 13 bactéries à Gram-positif dont les gènes ont été révélés par le séquençage de leur génome. L'étude des différentes bactéries à Gram-positif a montré que les CPS-A, à l'inverse des autres types de CPS, semblent peu fréquentes dans ces organismes. De plus, la présence d'une CPS-A s'accompagne toujours d'une CPS-P chez les bactéries étudiées (*L. plantarum*, *B. subtilis* et *B. stearothermophilus*). Une grande diversité a été mise en évidence quant au nombre de CPS présentes et à l'organisation de leurs gènes.

arginine / biosynthèse des pyrimidines / bactérie lactique / Gram-positif / carbamyl phosphate synthétase

1. INTRODUCTION

Carbamoyl-phosphate synthetases (CPS) catalyze the biosynthesis of carbamoyl phosphate (CP). CPS are allosteric heterodimeric enzymes composed of a small glutaminase (GLN) subunit encoded by *carA* (also called *pyrAa*) and a large synthetase (SYN) subunit encoded by *carB* (also called *pyrAb*). CPS are classified in three types according to their regulation and ability to utilize glutamine [2]. In most CPS, the GLN subunit desaminates glutamine into glutamate and the produced NH_3 group is transferred to the SYN subunit. In the CPS with a nonfunctional GLN subunit, the SYN subunit utilizes the NH_4^+ ions present in the media [2]. CP biosynthesis occurs in the SYN subunit in the presence of an NH_3 group, bicarbonate (the dissolved form of CO_2) and 2 ATP. The mechanism of CP synthesis is universal [2] and complex with several unstable intermediates [17, 18]. These labile compounds are protected by an intramolecular tunnel between the different active sites as shown by the X-ray crystal structure of the *E. coli* CPS [33].

CPS are well conserved and were the first enzymes used to create phylogenetic trees up to the progenote [21]. The existence of three different types of CPS [2], the presence of several CPS in some organisms, and the variation in CPS length, has inspired many hypotheses for CPS evolution which include gene duplication with subsequent domain specialization [2, 21, 36]. For instance, the SYN subunit has two homologous subdomains suggesting the duplication of an ancestral gene [29]. In *Aquifex aeolicus* [accession numbers AE000727 and AE000772] and *Methanococcus jannashii* [accession numbers AAB99385 and AAB99391] the two SYN subdomains are not linked and are present on different genes. This situation may represent an intermediate in CPS evolution where the two duplicated subdomains have not yet been joined in a SYN subunit as found in other species.

CP is a precursor of arginine and pyrimidines (Fig. 1) so that arginine-repressed

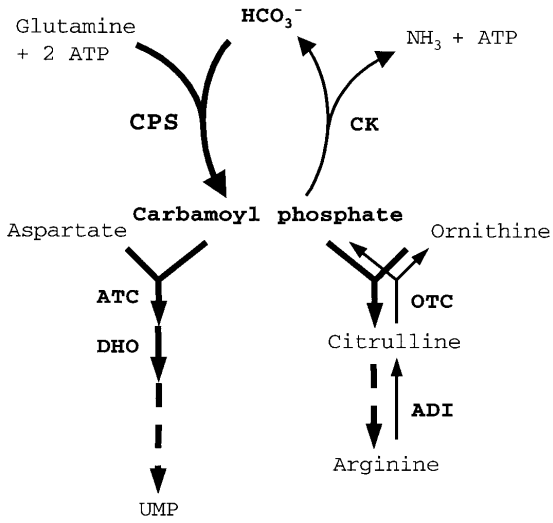

Figure 1. Carbamoyl phosphate, a common intermediate of arginine and pyrimidine biosynthesis. CPS, carbamoyl-phosphate synthetase; ATC, aspartate carbamoyl transferase; DHO, dihydroorotase. Enzymes of the arginine catabolism by the arginine deiminase pathway are ADI (arginine deiminase), OTC (catabolic ornithine carbamoyl transferase) and CK (carbamate kinase) and are indicated with thin arrows.

CPS (CPS-A), or pyrimidine-regulated CPS (CPS-P), or CPS regulated by both pathways can be found. Organisms have none, one or both types of CPS.

Eukaryotes harbour one or two CPS with a cellular compartmentalization (nucleus, cytosol or mitochondria) and often with tissue-specific distribution [2]. CPS-P in vertebrates are present in the multifunctional protein CAD which regroups three enzymatic activities involved in pyrimidine biosynthesis (CPS; ATC, aspartate carbamoyltransferase and DHO, dihydroorotase) (Fig. 1). A nuclear CAD-like protein is found in yeast with a nonfunctional DHO domain [32]. The CPS-A in vertebrates is mitochondrial and participates in the urea cycle in liver and in arginine biosynthesis in the small intestine [2, 36]. CPS-A in yeast is found either in the cytosol or mitochondria [11].

Few procaryotes, such as *Bacillaceae* and *L. plantarum*, have been shown to harbour a CPS-A ([28, 30, 37], Fig. 2). Prokaryotic CPS-A, but also some single CPS, are transcriptionally repressed by the binding of a repressor in presence of arginine to specific ARG-BOX [4, 13, 24, 28]. CPS-P are regulated by pyrimidine-dependent transcription attenuation with attenuator sites present in various configurations and numbers in different prokaryotes [14, 15, 26, 35 and possibly 19]. Binding of the allosteric effectors linked to the pyrimidine and the purine metabolisms has been located at the SYN carboxy terminal sequence [10, 37]. This domain was absent in the prokaryotic CPS-A [28, 37] and was linked to the fact that no allosteric effectors have been identified in CPS-A [30, 37].

In this paper, we focused on the diversity found in CP biosynthesis within lactic acid bacteria and we compared them to those of well-studied prokaryotes. Ten genera have been found in the lactic acid bacteria group, but only three genera (*Lactobacillus*, *Enterococcus* and *Lactococcus*) have been studied for their CP synthesis. We chose to study this pathway in *Lactobacillus* because we found that more than one third of the strains isolated from various fermented foods and environments are CP metabolism impaired since they are double auxotrophs for arginine and the pyrimidines (Bringel, personal communication). No auxotrophs for only the pyrimidines were found in our collection of 150 strains. In most cases, the auxotrophy was observed only in

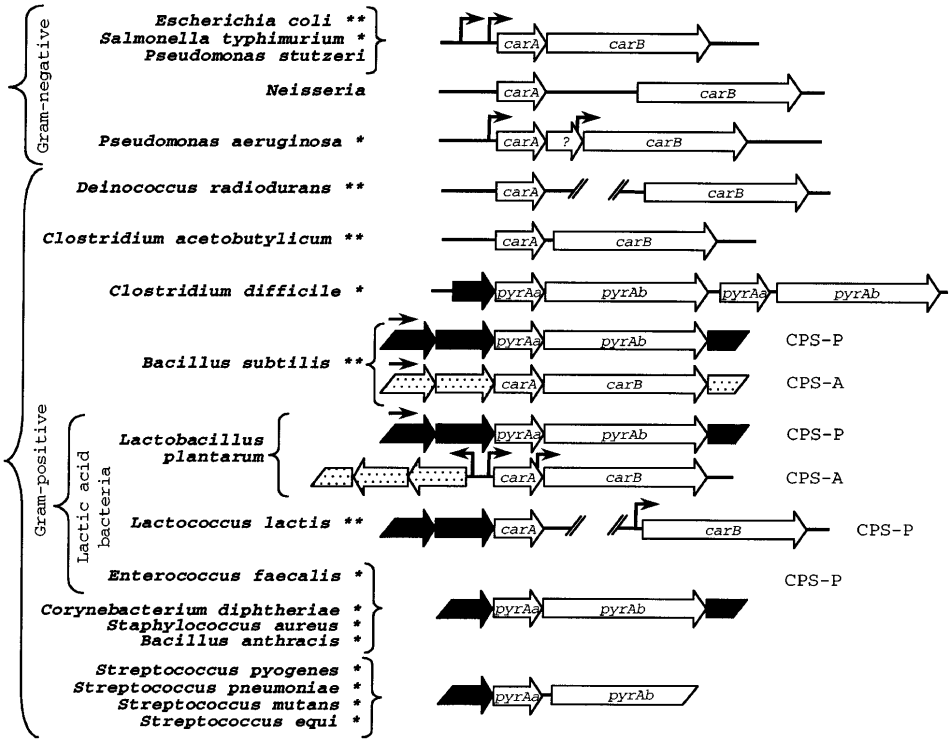

Figure 2. CPS gene organization within 19 prokaryotes. **, microorganisms completely sequenced; *, microorganisms whose genome is currently being sequenced. Arrows indicate gene orientation. Black genes are involved in the pyrimidine biosynthesis; granular genes are implicated in the arginine biosynthesis. Black arrows indicate the transcription direction or the initial point of transcription experimentally determined. CPS genes linked to the pyrimidine biosynthetic genes were designated *pyrAa* and *pyrAb* in *B. anthracis*, *C. difficile*, *C. diphtheriae*, *S. aureus* COL, *S. equi*, *S. mutans*, *S. pneumoniae* and *S. pyogenes*. On the other hand, the CPS genes of *C. acetobutylicum* and *D. radiodurans* were called *carA* and *carB*. The nature of the CPS (CPS-A or CPS-P) is indicated when it has been experimentally tested.

aerobiosis and was alleviated by the presence of CO_2 or anaerobiosis [6]. Moreover, CO_2 has an effect on CP biosynthesis in *Lactobacillus* [28]. In lactic acid bacteria, the arginine catabolism may lead to CP synthesis when the arginine deiminase (ADI) pathway is present. The ADI pathway involves three enzymatic steps schematized in Figure 1. The presence or absence of the ADI pathway in the studied strains will be used to discuss CP biosynthesis in lactic acid bacteria.

2. MATERIALS AND METHODS

The CPS-A sequence of *L. plantarum* (X99978) was used to search for potentially undescribed CPS which may be present in three Gram-positive bacteria whose genomes have been completely sequenced (*Bacillus subtilis*, *Clostridium acetobutylicum* and *Deinococcus radiodurans*) and nine Gram-positive bacteria with partially sequenced genomes (*Bacillus anthracis*, *Clostridium difficile*, *Corynebacterium diphtheriae*,

Enterococcus faecalis, *Staphylococcus aureus* COL, *Streptococcus mutans*, *Streptococcus equi*, *Streptococcus pneumoniae*, *Streptococcus pyogenes* Manfredo). To analyze the gene organization of these undescribed CPS, the putative functions of the surrounding ORFs were analyzed by homology with the protein data banks using the BLAST program [1]. Sequence analyses were performed using the Genetics Computer Group package from the University of Wisconsin [12b]. The ClustalX program [34] was used for protein alignments and construction of the phylogenetic tree with the undescribed and known eubacteria CPS (*B. caldolyticus* CPS-P (P46537); *B. stearothermophilus* CPS-A (AAC78719) and CPS-P (CAA05020); *E. coli* (P00968); *L. plantarum* CPS-P (Z54240.1); *Lc. lactis carB* (AJ000109)).

3. RESULTS AND DISCUSSION

3.1. Gram-negative bacteria CPS

Only one CPS has been found in Gram-negative bacteria but the genetic organization of the CPS genes varies in different microorganisms (Fig. 2). In *E. coli* and *Salmonella typhimurium*, the *carAB* operon has tandem promoters respectively controlled by arginine and the pyrimidines [5]. In *Neisseria*, the CPS genes are separated by 2.2 to 3.7 kb with repeated sequences [20]. This intergenic region could be implied in *carA* mutation but not in *carB* [20] resulting in CPS-deficiency in many *Neisseria* isolates. Unlike in *Pseudomonas stutzeri*, in *P. aeruginosa* the CPS genes are co-transcribed with a 682 bp ORF of unknown function [19] and *carB* is also monocistronically transcribed [23].

3.2. Gram-positive bacteria CPS

Genetic and biochemical studies of CP biosynthesis have been described in a few Gram-positive bacteria (*Bacillaceae* and four lactic acid bacteria). As more genomes are sequenced, more CPS sequences and their gene organizations will be found.

3.2.1. Bacillaceae

CP biosynthesis has been studied the most in *B. subtilis* which is prototrophic for the arginine and the pyrimidines. *B. subtilis* has two CPS [30]: CPS-A is encoded by the *carAB* genes which are part of the *argCJB-DcarABargF* operon repressed by arginine (Z26919 [4], Fig. 2); CPS-P is encoded by the *pyrAa* and *pyrAb* genes present on the pyrimidine biosynthetic operon *pyrRPB-CAaAbDIIDIFE* [31].

3.2.2. Lactic acid bacteria

3.2.2.1. Lactobacillus delbrueckii ssp. *lactis*

L. delbrueckii ssp. *lactis* needs exogenous arginine but not pyrimidines to grow. When C^{14}-radiolabeled arginine was given to this *Lactobacillus* [16], labelled pyrimidines were obtained demonstrating that arginine and not CO_2 (or bicarbonate) was the precursor of pyrimidines. No CPS is present in this *Lactobacillus* and the catabolism of arginine (via the ADI pathway) provides CP for pyrimidine biosynthesis.

3.2.2.2. Lactobacillus plantarum

Like *B. subtilis*, *L. plantarum* CCM 1904 is prototrophic for arginine and the pyrimidines and harbours two CPS. The difference in gene organization between the two microorganisms is summarized in Figure 2. No ADI pathway is present in strain CCM 1904. CPS-P genes are part of the *pyrRBCAaAbDFE* operon regulated by transcriptional attenuation [14]. CPS-A genes are part of an arginine-repressed cluster with the *carAB* operon being divergently transcribed compared to the *argCJBDF-ccl* operon [7]. In addition to its transcription within the *carAB* operon, *carB* is also

independently transcribed [28]. This situation has also been described in *P. aeruginosa* [28] (Fig. 2). In contrast to CPS-P, CPS-A is unable to provide CP for both pathways, as demonstrated by CPS deletion mutants of *L. plantarum* only harbouring one type of CPS [28]. CPS-A may be less active or less abundant than CPS-P.

CPS-A was shown to be dependent on higher CO_2 (or bicarbonate) concentrations. A mutant having only a CPS-A did not grow in defined media in presence of pyrimidines in ordinary air unless air was enriched with CO_2 [28]. Considering that CO_2 (or its dissolved form bicarbonate) is one of CPS substrates, two hypotheses are possible: *L. plantarum* CPS-A has a low affinity for bicarbonate; or its affinity is normal but the CO_2/bicarbonate level in *L. plantarum* is low. It is the first description of a natural CPS with higher CO_2 requirements. Conditional uracil-sensitive mutants have been described in *E. coli* and the molecular basis of the CO_2 growth stimulation observed in four of these mutants was correlated to amino acid substitutions within the large CPS subunit, causing lowered apparent affinity for substrate Mg-ATP or bicarbonate [12a].

Not only *L. plantarum* CCM 1904, but all of the 90 prototrophic *L. plantarum* strains tested in the laboratory are uracil-sensitive in aerobiosis and not when incubated in CO_2-enriched air. This was also observed for related species such as *L. pentosus* and *L. paraplantarum*. Since CPS-P is inhibited in the presence of uracil and CP synthesis relies on CPS-A, the higher CO_2-dependency of CPS-A than of CPS-P seems to be a general feature in these lactobacilli species. Thus CPS-A activity in these mesophilic homo-fermentative lactobacilli depends on CO_2 concentrations in natural fermentations and in particular on CO_2-producing microorganisms (hetero-fermentative lactic acid bacteria, yeast, etc.) which coexist in these biotopes.

3.2.2.3. Lactococcus lactis

Lc. lactis MG 1363 and IL 1403 are both prototrophic for the pyrimidines but only MG 1363 is prototrophic for arginine [8, 26]. The occurrence of a unique CPS in *Lc. lactis* was demonstrated by IL 1403 complete sequenced genome [3] and genetically with a MG 1363 *carB* mutant [26]. The *carA* and *carB* genes are dispersed in the *Lc. lactis* chromosome ([3, 26], Fig. 2). Transcription of both genes are controlled by a pyrimidine attenuation mechanism. The *carA* gene seems to be part of the *pyrRPB-carA* operon and *carB* is transcribed monocistronically [3, 26].

The MG 1363 *carB* mutant with no functional CPS grows without pyrimidine when exogenous arginine is provided. Like in *L. delbrueckii* ssp. *lactis*, arginine catabolism via the ADI pathway provides CP for pyrimidine biosynthesis [26]. Thus, arginine metabolism influences the cellular CP pool and may regulate pyrimidine biosynthesis in this strain. In the absence of arginine, CPS-P alone provides CP for arginine and pyrimidine biosynthesis in MG 1363.

Even though IL 1403 is auxotrophic for arginine, the regulatory and the structural genes involved in arginine biosynthesis were found in the chromosome [3] suggesting minor genetic lesions in one of the corresponding genes (*carA*, *carB*, *argCJDBF*, *ahrC* or *argGH*). Thus, CPS-P might not be functional in this strain and its activity should be tested.

3.2.2.4. Enterococcus faecalis

Arginine auxotrophy (or bradytrophy) and pyrimidine prototrophy are commonly found in *E. faecalis* [22, 27]. Genes encoding CPS-P are present on the *pyrRPB-CAaAbKDFE* operon and are pyrimidine-regulated by transcriptional attenuation at a single site in the 5' leader [15]. This CPS-P is functional and able to complement an *E. coli* CPS-deficient mutant [22]. The presence of CPS-A in *E. faecalis* is unlikely since the strain is auxotrophic for arginine

and since no second CPS was found in the nearly completely sequenced genome. The ADI pathway is often present [25] but natural ADI defective variants have also been described [9]. As found in *Lc. lactis*, when the ADI is present it may provide CP for pyrimidine biosynthesis, but we have found no data to support this.

3.2.3. Analysis of 20 sequenced Gram-positive bacteria CPS

We searched for potentially undescribed CPS which may be present in completely or partially sequenced genomes of *B. anthracis*, *B. subtilis*, *C. acetobutylicum*, *C. difficile*, *C. diphtheriae*, *D. radiodurans*, *E. faecalis*, *S. aureus* COL, *S. equi*, *S. mutans*, *S. pneumoniae* and *S. pyogenes* Manfredo (see Materials and Methods). The results obtained with the Gram-positive bacteria are shown in Figure 2.

In order to know the nature of these undescribed CPS (CPS-P, CPS-A or CPS regulated by both pathways), we analyzed their genetic context. The presence of genes involved in pyrimidine biosynthesis near some undescribed CPS suggested that *B. anthracis*, *C. difficile*, *C. diphtheriae*, *S. aureus* COL, *S. equi*, *S. pyogenes* Manfredo, *S. pneumoniae* and *S. mutans* harbour a CPS-P (Fig. 2). In *C. difficile*, two CPS genes are linked and preceded by a gene involved in pyrimidine biosynthesis (*pyrF*, coding for the OMP decarboxylase). Within pyrimidine-regulated CPS, the C-terminal end of the SYN subunit has been shown to bind allosteric effectors [10] and the two CPS found in *C. difficile* have very conserved C-terminal sequences (data not shown), confirming the CPS-P nature of these enzymes. The two CPS present in *C. difficile* are homologous (with 72% and 93% identity between their GLN and SYN subunits respectively) suggesting a gene duplication in this organism. When the sequence of this organism will be completed, the presence of more than two CPS in *C. difficile* can be checked. No genes linked to the pyrimidine or arginine pathways were found either for *C. acetobutylicum* or *D. radiodurans*. For further characterization, phylogenetic trees were constructed with the small GLN and the large SYN subunits of undescribed CPS and characterized eubacteria CPS (see Materials and Methods). The known Gram-positive CPS-P of *Lc. lactis*, *E. faecalis*, *L. plantarum*, *B. subtilis*, *B. stearothermophilus* and *B. caldolyticus* were present in a branch which also contained the CPS of *B. anthracis*, *S. aureus* COL, *S. equi*, *S. pyogenes* Manfredo, *S. pneumoniae* and *S. mutans* (data not shown) confirming the CPS-P nature of these enzymes. On the other hand, the two CPS of *C. difficile* and the CPS of *C. acetobutylicum* were not found in the CPS-P and CPS-A branches. *C. diphtheriae* and *D. radiodurans* CPS were found in the *E. coli* CPS group. Of the 20 Gram-positive bacteria CPS currently available, only three are linked to arginine biosynthetic genes and are CPS-A (*L. plantarum*; *B. subtilis* and *B. stearothermophilus*) [28, 30, 37]. Thus, CPS-A are less frequent than CPS-P in Gram-positive bacteria.

4. CONCLUSION

The study of CP biosynthesis in different organisms depicts a striking diversity in the number of CPS (from none to two), their organization, their regulation and physiological role. The described CPS gene organization represent certainly a small range of the situations found in Gram-positive bacteria and CPS evolution appears to be quite complex. Among 17 Gram-positive bacteria, 9 different sets of CPS genes were encountered (Fig. 2 which does not include *L. delbrueckii* ssp. *lactis*, *B. caldolyticus* and *B. stearothermophilus*). This diversity was also found within the lactic acid bacteria studied (*L. plantarum*, *L. delbrueckii* ssp. *lactis*, *E. faecalis* and *Lc. lactis*) and revealed that there is no simple model for CP synthesis in this bacterial group. CPS-A

were not often found in prokaryotes and when they were present, a CPS-P was also present (*B. subtilis*, *B. stearothermophilus* and *L. plantarum*).

The occurrence of *Neisseria* CPS-deficient variants has been linked to the presence of an intergenic insert with repeated sequences between *carA* and *carB* [20]. In *L. plantarum* , CPS may also be implied in the occurrence of the natural CO_2-conditional double auxotrophs for arginine and the pyrimidines. However, the CPS of a prototrophic *L. plantarum* has been sequenced [28], the gene organization was studied but no IS, transposons or repeated sequences have been found.

The impact of the ecological niche and the effect of CO_2 on *L. plantarum* CPS in particular have yet to be determined.

ACKNOWLEDGMENTS

Sequence data for *C. difficile*, *C. diphtheriae*, *S. equi* and *S. pyogenes* were produced by the *C. difficile*, *C. diphtheriae*, *S. equi* and *S. pyogenes* Sequencing Groups at the Sanger Centre and can be obtained from ftp://ftp.sanger.ac.uk/pub/ pathogens/cd/, ftp://ftp.sanger.ac.uk/pub/pathogens/cdip/, ftp://ftp.sanger.ac.uk/pub/pathogens/se/ and ftp://ftp.sanger.ac.uk/pub/pathogens/sp/. Preliminary sequence data for *B. anthracis*, *S. aureus* COL and *S. pneumoniae* were obtained from The Institute for Genomic Research (TIGR) website at http://www.tigr.org. These organisms were sequenced with the help of the TIGR, the Merck Genome Research Institute, the National Institute of Allergy and Infectious Disease, the Office of Naval Research and the Department of Energy (DOE). Preliminary sequence data for *S. mutans* was obtained from the Genome Center at the University of Oklahoma (OU-ACGT) website at http://www.genome.ou.edu/smutans.html. The *S. mutans* genome sequencing project was funded by a USPHS/NIH grant from the Dental Institute.

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