Partial redundancy in the synthesis of the D-arabinose incorporated in the cell wall arabinan of *Corynebacterineae*

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The major cell wall carbohydrate of *Corynebacterineae* is arabinogalactan (AG), a branched polysaccharide that is essential for the physiology of these bacteria. Decaprenylphosphoryl-D-arabinose (DPA), the lipid donor of D-arabinofuranosyl residues of AG, is synthesized through a series of unique biosynthetic steps, the last one being the epimerization of decaprenylphosphoryl- β -D-ribose (DPR) into DPA, which is believed to proceed via a sequential oxidation–reduction mechanism. Two proteins from *Mycobacterium tuberculosis* (Rv3790 and Rv3791) have been shown to catalyse this epimerization in an *in vitro* system. The present study addressed the exact function of these proteins through the inactivation of the corresponding orthologues in *Corynebacterium glutamicum* (NCgl0187 and NCgl0186, respectively) and the analysis of their *in vivo* effects on AG biosynthesis. We showed that NCgl0187 is essential, whereas NCgl0186 is not. Deletion of NCgl0186 led to a mutant possessing an AG that contained half the arabinose and rhamnose, and less corynomycolates linked to AG but more trehalose mycolates, compared with the parental strain. A candidate gene that may encode a protein functionally similar to NCgl0186 was identified in both *C. glutamicum* (NCgl1429) and *M. tuberculosis* (Rv2073c). While the deletion of NCgl1429 had no effect on AG biosynthesis of the mutant, the gene could complement the mycolate defect of the AG of the NCgl0186 mutant, strongly supporting the concept that the two proteins play a similar function *in vivo*. Consistent with this, the NCgl1429 gene appeared to be essential in the NCgl0186-inactivated mutant. A detailed bioinformatics analysis showed that NCgl1429, NCgl0186, Rv3791 and Rv2073c could constitute, with 52 other proteins belonging to the actinomycetales, a group of closely related short-chain reductases/dehydrogenases (SDRs) with atypical motifs. We propose that the epimerization of DPR to DPA involves three enzymes that catalyse two distinct steps, each being essential for the viability of the bacterial cells.

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Abbreviations: AG, arabinogalactan; Ara, arabinose; Ara_f, arabinofuranosyl; DPA, decaprenylphosphoryl-D-arabinose; DPPR, decaprenyl-monophosphoryl-5-phosphoribose; DPR, decaprenyl-monophosphoryl-D-ribose; Gal, galactose; mAGP, mycolyl-arabinogalactan-peptidoglycan; PAD, pulsed amperometric detector; ppRp, 5-phosphoribofuranose pyrophosphate; Rha, rhamnose; SDR, short-chain dehydrogenase/reductase; Suc, sucrose; TDCM, trehalose dicorynomycolate; TFA, trifluoroacetic acid; TCM, trehalose monocorynomycolate.

Two supplementary tables, listing the primers used in this study and the orthologous group of proteins obtained by computational analysis using protein sequence alignments of strains examined in this study, are available with the online version of this paper.

INTRODUCTION

The *Corynebacterineae* is a suprageneric actinomycetes group that includes the causative agents of the human diseases tuberculosis, leprosy and diphtheria, *Mycobacterium tuberculosis*, *Mycobacterium leprae* and *Corynebacterium diphtheriae*, respectively. The cell wall core of these bacteria is made up of an unusual matrix composed of a cross-linked peptidoglycan covalently bound to arabinogalactan (AG) chains, which in turn are linked to mycolic acids, allowing the attached lipids to form the inner leaflet of an outer membrane. This complex is often referred to as mycolyl-arabinogalactan-peptidoglycan (or mAGP) complex (Daffé & Draper, 1998).

Structural motifs of the AG polymer were first established for *M. tuberculosis* (Daffé *et al.*, 1990). However, except for some variations in glycosyl linkage compositions, notably in the arabinosyl portion, the major structural features of AG are conserved among all *Corynebacterineae* examined so far (Daffé *et al.*, 1993). In mycobacteria the polysaccharide is composed of two distinct regions: a homogalactan portion, composed of alternating 5-linked and 6-linked β -galactofuranosyl residues (Daffé *et al.*, 1990, 1993), which is covalently linked to the peptidoglycan via a disaccharide phosphate (McNeil *et al.*, 1990), and arabinan chains, formed from arabinofuranosyl (Araf) residues, attached to carbon 5 of some of the 6-linked galactosyl (Gal) residues (Daffé *et al.*, 1990) at their reducing termini (Besra *et al.*, 1995) and esterified with mycolic acids at their non-reducing termini (McNeil *et al.*, 1991). In addition to galactose (Gal) and arabinose (Ara), rhamnose (Rha) is also found in AG of *Corynebacterium glutamicum* (Alderwick *et al.*, 2005; Gebhardt *et al.*, 2007).

In the last few years an increasing number of studies have shown that *C. glutamicum* constitutes a good model for investigating biosynthetic pathways of specific cell wall compounds of *Corynebacterineae*, such as mycolic acids and AG. Indeed, while these components are essential for the viability of mycobacteria, *C. glutamicum* mutants either totally or severely deprived of mycolates or synthesizing a truncated AG devoid of the arabinan domain are viable, although they grow slowly (Alderwick *et al.*, 2005, 2006b; Portevin *et al.*, 2004, 2005; Tropis *et al.*, 2005).

The synthesis of the arabinan domain of AG results from the sequential addition of Araf residues to the galactan domain by specialized arabinosyltransferases such as Embs (Alderwick *et al.*, 2005; Escuyer *et al.*, 2001), AftA (Alderwick *et al.*, 2006b) and AftB (Seidel *et al.*, 2007). These enzymes utilize the unusual sugar donor decaprenylphosphoryl-D-arabinose (DPA) (Alderwick *et al.*, 2005; Wolucka *et al.*, 1994; Xin *et al.*, 1997), which has been shown to be the only donor for the Araf residues in both mycobacteria and corynebacteria. It has been demonstrated recently that DPA is synthesized from phosphoribose diphosphate (pRpp) (Scherman *et al.*, 1996) through an unusual series of three successive reactions (Mikusova *et al.*, 2005): (i) transfer of pRpp to decaprenyl phosphate to

form decaprenylphosphoryl-5-phosphoribose (DPPR); (ii) removal of the 5' phosphate giving decaprenylphosphoryl ribose (DPR); and (iii) epimerization of DPR to DPA, which likely occurs via a sequential oxidation-reduction mechanism involving an intermediate (DPX), product of DPR oxidation and precursor of DPA (Fig. 1). Although not unambiguously identified, DPX is probably a decaprenylphosphoryl-2-keto- β -D-erythro-pentofuranose.

The only enzyme identified in the DPA biosynthesis pathway by gene inactivation and mutant analysis is the DPPR synthase, the product of the essential *M. tuberculosis* gene *Rv3806c* (Huang *et al.*, 2005), and named UbiA in *C. glutamicum* (Alderwick *et al.*, 2005). Inactivation of *ubiA* in this bacterium produces a mutant totally devoid of arabinan (Alderwick *et al.*, 2005). Very recently, on the basis of the similarity with proteins implicated in the arabinosylation of the *Azorhizobium caulinodans* nodulation factor, Mikusova *et al.* (2005) have proposed that *M. tuberculosis* genes *Rv3790* and *Rv3791*, which are both annotated as probable oxidoreductases, could encode the two enzymes expected to be involved in the epimerization of DPR into DPA. The two genes have been cloned and expressed in *Escherichia coli*, and the corresponding proteins purified. The use of an *in vitro* assay has shown that the presence of both proteins is required to transform DPR to DPA, suggesting that the two proteins work in concert to catalyse the epimerization. It was thus interesting to address the question of the precise role of these enzymes in the biosynthesis of AG *in vivo*. As *Rv3790* and *Rv3791* are presumably essential in *M. tuberculosis* (Sasseti *et al.*, 2003) we attempted to delete their *C. glutamicum* orthologues, *NCgl0187* and *NCgl0186*, respectively, and characterize the resulting AG modifications.

METHODS

Strains and culture conditions. *C. glutamicum* (ATCC 13032 RES167) (Dusch *et al.*, 1999) was cultured on Brain Heart Infusion (BHI) medium (Difco) at 30 °C. *E. coli* DH5 α and Top10 (Invitrogen) were used for the construction of plasmids and were grown on Luria-Bertani (LB) medium (Difco) at 37 °C. Ampicillin, kanamycin (Km), chloramphenicol (Cm) and sucrose (Suc) were added when required at final concentrations of 10, 25, 30 $\mu\text{g ml}^{-1}$ and 10% (w/v) (for *E. coli*), or 6 $\mu\text{g ml}^{-1}$ and 10% (w/v) (for *C. glutamicum*), respectively. Transformation of *C. glutamicum* by electroporation was performed as described by Bonamy *et al.* (1990).

DNA manipulations. *C. glutamicum* chromosomal DNA was extracted as described by Ausubel *et al.* (1987). Oligonucleotide primers were synthesized by Genosys. All DNA sequencing was carried out by Cogenics.

Construction of plasmids and strains. In order to delete *NCgl0186* or *NCgl0187*, we used the strategy described by Schafer *et al.* (1994). In brief, two DNA fragments overlapping the gene to be deleted at its 5' and 3' extremities were amplified by PCR from *C. glutamicum* total DNA by using appropriate primers (186-1/186-2 and 186-3/186-4 for *NCgl0186*, and 187-1/187-2 and 187-3/187-4 for *NCgl0187*; see Supplementary Table S1) and cloned in the non-replicative vector pK18mobSac. The resulting plasmids (pK18mobsac Δ 186 and

Fig. 1. The biosynthetic pathway proposed for DPA synthesis from pRpp (Mikusova *et al.*, 2005). The isoprenoid moiety is drawn to conform to the structure of decaprenyl phosphate identified in mycobacteria. The structure of the corynebacterial equivalent is not known. Although the structure of DPX is not known with certainty, this intermediate is very likely a decaprenylphosphoryl-2-keto-β-D-erythropentofuranose. The role of NCgl2782 (Rv3807c) in the dephosphorylation step is putative and arises from the position of the gene on the chromosome, just beside *ubiA* (Rv3806c), and from its annotation as a phosphatase.

pK18mobsacΔ187) were sequenced and independently transferred into *C. glutamicum* ATCC 13032 RES167 by electroporation. Transformants in which the construct was integrated into the chromosome by single crossing over were selected on BHI plates containing Km. The second crossover event was selected by plating Km^R clones on BHI plates containing Suc. Km^S and Suc^R colonies were screened by PCR for the correct deletion of the gene using primers binding upstream and downstream of the NCgl0186 sequence. After verification of PCR products by sequencing, one strain carrying the NCgl0186 deletion (Cg-Δ186) was selected for further studies.

Construction of a mutant strain carrying a deletion of the NCgl0187 gene was not successful. We thus tried to inactivate this gene. For this purpose, an internal fragment was amplified using primers 187-5 and 187-6 (see Supplementary Table S1) and cloned into plasmid pCR 2.1-TOPO using the TOPO TA cloning kit (Invitrogen) to give plasmid TOPO::187. This plasmid was used to electrotransform the ATCC 13032 RES167 strain. No transformant carrying the correct plasmid integration could be detected after selection on BHI plates containing Km and PCR analysis.

Deletion of the NCgl1429 gene was done using a strategy described previously (Portevin *et al.*, 2004). In brief, two DNA fragments

overlapping the NCgl1429 gene at its 5' and 3' extremities were amplified by PCR from *C. glutamicum* total DNA using primers 1429-AMXho/1429-AMSac and 1429-AVSac/1429-AVBgl, respectively (Supplementary Table S1). These fragments were inserted flanking a *km* resistance cassette into plasmid pMCS5 (MoBiTec). The resulting plasmid was transferred into *C. glutamicum* ATCC 13032 RES167 by electroporation, and transformants were selected on plates containing Km. Transformants in which allelic replacement had occurred were selected by PCR analysis using combinations of primers binding upstream and downstream of NCgl1429 and in the *aphIII* sequences. After sequencing of the PCR products, one strain (Cg-Δ1429) was selected for further studies.

For inactivation of NCgl1429 in Cg-Δ186, two plasmids were constructed. First, an internal fragment was amplified using primers 1429-1 and 1429-2 (see Supplementary Table S1) and cloned into plasmid pCR2.1-TOPO to give plasmid TOPO::1429. Then, a fragment corresponding to the entire NCgl0186 ORF under the *cpb* promoter was amplified from plasmid pCGL2330 (see below), digested with *HindIII* and *SpeI*, and ligated to TOPO::1429 digested with the same restriction enzymes. The resulting plasmids, TOPO::1429-cspB186 and TOPO::1429, were both used to electrotransform strain Cg-Δ186, and transformants were selected

on BHI plates containing Km. Genomic DNAs from mutants were analysed by three PCR amplifications using two primers binding upstream of the 5' end and downstream of the 3' end of the *NCgl1429* gene, and the M13 forward and reverse primers binding in the pCR2.1-TOPO vector. The PCR products corresponding to the gene disruption borders were systematically sequenced.

Expression vectors encoding NCgl0186 (pCGL2330), Rv3791 (pCGL2331) and NCgl1429 (pCGL2333) were constructed using pCGL482 (Peyret *et al.*, 1993) as the cloning vector. We chose to clone these three ORFs under the control of the *csfB* promoter (Peyret *et al.*, 1993). Coding sequences from *C. glutamicum* ATCC 13032 chromosomal DNA were amplified by PCR using primer pairs 187-3/186-7 for NCgl0186 and 1429-3/1429-4 for NCgl1429. The Rv3791 ORF was amplified using primers 3791-1 and 3791-2 from the pSTblue1-Rv3791 plasmid (Mikusova *et al.*, 2005). A DNA fragment containing the *csfB* promoter was generated using primers pscfBBam and pscfBNco and pCGL824 (Peyret *et al.*, 1993) as template DNA. All the amplicons were digested with the appropriate restriction endonucleases (see Supplementary Table S1). Plasmids were obtained by simultaneous ligation of fragments containing the *csfB* promoter, fragments containing one ORF and the appropriately digested pCGL482. Transformants were selected on Cm-containing plates.

AG preparation. Cell walls were prepared as described previously (Daffé *et al.*, 1990). Briefly, wet cells were resuspended in PBS buffer (50 mM, pH 7.2) at the concentration of 5 g in 20 ml and broken in a cell disrupter (2.7×10^8 Pa). After three cell disrupter cycles, intact cells were removed by centrifugation (3000 g) and the supernatant containing the cell walls was then centrifuged (27 000 g, 1 h). The resulting cell wall pellet was treated with aqueous 2% (w/v) SDS at 95 °C for 1 h and sedimented again at 27 000 g. This was followed by two acetone washes and the pellet thus obtained was mAGP. mAGP was further treated with 2 M NaOH and stirred gently at 80 °C for 16 h to remove the mycolic acid residues and cleave peptidoglycan to solubilize AG. The pellet was discarded and the pH of the supernatant was adjusted to 7.0 with glacial acetic acid. This neutralized AG was dialysed for at least 3–4 h with three 1 l changes of MilliQ water. The AG obtained was concentrated by rotary evaporation to a final volume of 1 ml, and was freeze-dried and stored till further use.

Enzymic treatments of soluble AG. The soluble AG was treated with partially purified endogenous arabinase (Dong *et al.*, 2006) at 37 °C for 12 h, followed by extraction with CHCl₃/CH₃OH/H₂O (10:10:3, by vol.). After centrifugation, the supernatant, which contained most of the released arabinans, was used for MALDI-TOF MS analysis.

To further elucidate the cell wall AG configuration, the soluble AG was treated with *Cellulomonas* arabinase obtained as described previously (McNeil *et al.*, 1994), which specifically releases Ara2 and Ara4 from AG.

Analysis of AG. The glycosyl composition of purified cell walls was determined by hydrolysing an aliquot of mAGP with 2 M trifluoroacetic acid (TFA) for 2 h at 110 °C, followed by trimethylsilylation (Sweeley *et al.*, 1963) and GC analysis of the resulting products, or by alditol acetate formation, as described previously (Daffé *et al.*, 1990).

HPLC analysis of soluble AG samples treated with arabinase was carried out on a Dionex system equipped with a CarboPac PA1 analytical column (4 × 250 mm). Total run time was 50 min, the NaOH concentration (100 mM) being consistent throughout. Sodium acetate concentration was varied from 0 to 450 mM for a period of 50 min. The eluted samples were detected using a pulsed amperometric detector (PAD).

For MALDI-TOF MS analysis, samples were peracetylated with acetic anhydride (100 µl) in the presence of pyridine (100 µl) at room temperature overnight and recovered after removal of the solvent in chloroform prior to MALDI-TOF MS. The acetylated AG samples were run in positive ion reflector mode on a Bruker Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonics). One microlitre of the sample was mixed with 1 µl matrix (10 mg ml⁻¹ dihydroxybenzoic acid in 50% acetonitrile/0.1% TFA) and allowed to air dry on the MALDI target plate.

For ¹H- and ¹³C-NMR analysis, AGs were solubilized in ²H₂O (20 mg in 0.5 ml) and analysed at 600 and 150.9 MHz, respectively, on a Bruker Avance 600 MHz NMR spectrometer equipped with a TCI cryoprobe. ¹H-decoupled ¹³C spectra were obtained with a DEPT 135 pulse sequence in a DQD acquisition mode.

Extraction and quantification of corynomycolic acids. Lipids were extracted from wet cells for 16 h with CHCl₃/CH₃OH (1:2, v/v) at room temperature; the cells were re-extracted with CHCl₃/CH₃OH (1:1, v/v) and CHCl₃/CH₃OH (2:1, v/v) for 16 h. The three organic phases were pooled and concentrated by means of rotary evaporation. The crude lipid extracts were partitioned between the aqueous and the organic phases arising from a mixture of CHCl₃/H₂O (1:1, v/v). The lower organic phases were collected and evaporated to dryness to yield the crude lipid extracts from each strain. Subsequently, they were examined comparatively using TLC on silica gel-coated plates (Durasil-25, 0.25 mm thickness, Macherey-Nagel) developed with CHCl₃/CH₃OH/H₂O (30:8:1 or 65:25:4, by vol.). Glycolipids were detected by spraying plates with 0.2% anthrone in concentrated H₂SO₄, followed by heating. The corynomycolate content of extractable lipids was determined in three independent experiments as follows. Lipid extracts (100 mg) of the various strains were dried under vacuum and saponified (Daffé *et al.*, 1983); the saponified products were acidified with 20% H₂SO₄. The resulting fatty acids were extracted with diethyl ether, washed with water, converted to methyl esters with diazomethane and dried under vacuum, and weighed. The fatty acid methyl esters were separated from contamination on a silica gel column irrigated with different concentrations of diethyl ether in petroleum ether (0, 5, 10, 20 and 100%, v/v). Fractions were analysed by TLC developed with CH₂Cl₂. Lipids were detected by spraying plates with rhodamine B and fractions containing corynomycolates were pooled and weighed. The AG-bound mycolic acids present in the delipidated cells, i.e. the material after the CHCl₃/CH₃OH extraction (described above), were saponified with 40% KOH in 2-methoxyethanol (1:7, v/v) at 110 °C for 3 h (Daffé *et al.*, 1983) and the solution was neutralized using 20% H₂SO₄. The released mycolic acids were extracted with diethyl ether and methylated with diazomethane. The resulting fatty acid methyl esters were examined using TLC on silica gel-coated plates developed as described above. When significant amounts of fatty acids other than mycolic acids were present in the saponification products, corynomycolates were isolated by chromatography on a silica gel column as described above and weighed.

Epimerase assay. The cell wall or cell membrane (70 µl, 5.5 mg protein ml⁻¹), 10 mM ATP (1 µl), 10 mM each of FAD, NAD and NADP (3 µl each), and [¹⁴C]pRpp (20 µl, 10 000 c.p.m. µl⁻¹) were added to MOPS buffer (pH 8 with MgCl₂) to give a final volume of 160 µl. The reaction mixture was incubated for 90 min at 37 °C. Subsequently, the mixture was extracted with CHCl₃/CH₃OH/H₂O (8:4:3, by vol.), centrifuged briefly at 4500 g, and the organic layer was separated and dried. This was subjected to TFA (2 M) hydrolysis for 1 h at 110 °C, cooled, dried down in CH₃OH and analysed for Ara and ribose content by HPLC. The samples (neutral sugars after TFA hydrolysis) were analysed using a CarboPac PA01 analytical column (4 × 250 mm). The sugars were separated with an isocratic solvent system consisting of 10% NaOH (100 mM) in water for 45 min. The

^{14}C -containing components were detected using a Beta ram detector. The same HPLC system was connected to a PAD to detect non-radioactive standards of the respective sugars for comparative quantification.

RESULTS

Genomic comparison of the 3790–3791 locus

Data from Mikusova *et al.* (2005) strongly suggest that in *M. tuberculosis* the products of *Rv3790* and *Rv3791* work in concert to catalyse the conversion of DPR to DPA. *Rv3790* and *Rv3791* are located upstream of the genes encoding the arabinosyltransferases *AftA*, *EmbA*, *EmbB* and *EmbC* (Fig. 2). All these genes are part of a large cell wall biosynthesis cluster containing, among others, *Rv3806c*, which encodes the enzyme involved in the first step of DPA synthesis, the transfer of a 5-phosphoribosyl residue from pRpp to decaprenyl phosphate to form DPPR (Huang *et al.*, 2005). An *in silico* analysis revealed that orthologues of *Rv3790* and *Rv3791* are present in all the available genome sequences of *Corynebacterineae*. The two genes are always organized in a putative operon and, except in *Corynebacterium jeikeium*, are adjacent to the *aftA-emb* cluster (Fig. 2). In *C. glutamicum*, *Rv3790* and *3791* orthologues correspond to genes *NCgl0187* and *NCgl0186*, respectively. Sequence comparisons at protein level show 65 % identity for *Rv3790* and *NCgl0187* and 57 % identity for *Rv3791* and *NCgl0186*. As *Rv3790* and *Rv3791* are presumably essential in *M. tuberculosis* (Sassetti *et al.*, 2003), attempts were made to delete their *C. glutamicum* orthologues in order to address the question of the functions of the corresponding enzymes in the biosynthesis of AG *in vivo*, a strategy already successfully used to decipher the functions of mycobacterial essential genes (Alderwick *et al.*, 2006a, b; Portevin *et al.*, 2004, 2005).

Construction and growth characteristics of Cg-Δ186

In an attempt to delete either *NCgl0186* or *NCgl0187*, non-replicative pK18mobsac-derivative plasmids were constructed (pK18mobsacΔ186 and pK18mobsacΔ187), each carrying sequences adjacent to the gene to be deleted (Schafer *et al.*, 1994). Km^{S} and Suc^{R} clones resulting from two recombination events between plasmid and chromosome were obtained for both deletions. In the case of *NCgl0186*, 50 clones were analysed by PCR and half of them were found to have a gene deletion at the expected position. In comparison to the wild-type strain, however, the Δ*NCgl0186* mutant (thereafter named Cg-Δ186) exhibited no significant phenotypic changes, had similar growth and did not aggregate in liquid cultures. These results indicated that although *NCgl0186* may be involved in the synthesis of DPA, the mutant strain has a behaviour very different from that previously observed for the mycolate-less or AG-truncated mutants, i.e. a rough and matt colony phenotype, instead of the smooth and shiny one of the wild-type, and very slow growth and strong aggregation in liquid cultures (Alderwick *et al.*, 2006a, b; Portevin *et al.*, 2004, 2005).

In the case of *NCgl0187* about 200 clones were analysed by PCR but, unlike *NCgl0186*, all of the clones showed the wild-type genotype, indicating either a strong disadvantage of *NCgl0187* deletion or the essentiality of the gene even in the corynebacterial host. Furthermore, even when the analysis was continued up to 3 weeks to eventually obtain slow-growing rough-textured colonies as described for *emb* and *aftA* mutants in *C. glutamicum* (Alderwick *et al.*, 2005, 2006b), no such colonies were visible. As no deletion mutant could be obtained for *NCgl0187*, we attempted to inactivate the gene by plasmid integration, but no integration mutants could be obtained by this procedure

Fig. 2. Comparison of the *Rv3790/3791* locus within *Corynebacterineae*. In *M. tuberculosis* the locus contains *Rv3790*, *Rv3791*, *aftA*, *embC*, *embA* and *embB*, and five other ORFs, three of unknown function and the last two annotated *fadD35* and *transposase*. Together these ORFs cover the genome sequence (RefSeq no. NC_000962) between positions 4 234 780 and 4 254 327. In *C. glutamicum* the locus contains *NCgl0186* (*Rv3790* orthologue), *NCgl0187* (*Rv3791* orthologue), *aftA*, *Cg-emb* (*embC* orthologue) and two ORFs of unknown functions. These ORFs cover the genome sequence (RefSeq no. NC_003450) between positions 201 757 and 211 759.

either. We thus concluded that *NCgl0187* is likely an essential gene.

Glycosyl compositional analysis of the cell wall and AG from Cg-Δ186 and ATCC 13032 strains

Purified cell walls from Cg-Δ186 and its parental ATCC 13032 wild-type of *C. glutamicum* were analysed for glycosyl composition. As shown in Table 1, Cg-Δ186 still produced Ara but contained only half of the amount observed in the wild-type strain, resulting in a lower Ara:Gal ratio than that of the ATCC 13032 strain. A similar diminution of the Rha:Gal ratio was also observed for the mutant strain, suggesting that the rhamnosyl residues are linked to the arabinan moiety of AG. Confirmation of these data came from the analysis of AG released from the peptidoglycan using base treatment, as described previously for mycobacterial cell walls (Daffé *et al.*, 1990), which showed a twofold decrease in the Ara:Gal and Rha:Gal ratios of the mutant when compared with those of the parental strain (data not shown). Complementation of Cg-Δ186 with plasmid pCGL2330 (containing *NCgl0186*) or pCGL2331 (containing *Rv3191*) led in both cases to the restoration of a normal level of Ara (Table 1). This indicated that the mycobacterial *Rv3191* protein exhibited *in vivo* a function similar to that of the corynebacterial *NCgl0186*. The Rha:Gal ratio was, however, only slightly increased (10%) by complementation of Cg-Δ186 with the plasmid containing *NCgl0186* and unchanged by the presence of a plasmid containing *Rv3791*, a phenomenon likely to be due to a problem with co-regulation of the synthesis of the Rha-containing arabinan segment of AG. We thus concluded that *NCgl0186* is directly or indirectly involved in the reaction of epimerization of DPR into DPA, but that another enzyme possessing a similar function is likely to be present in *C. glutamicum*. These data are consistent with the normal growth phenotype observed for the Cg-Δ186 strain.

Table 1. Quantitative analysis of the sugar constituents of the cell walls extracted from *C. glutamicum* ATCC 13032, its isogenic Δ*NCgl0186* (Cg-Δ186) mutant and the mutant complemented with either the corynebacterial gene [Cg-Δ186(pCGL2330)] or its mycobacterial orthologue [Cg-Δ186(pCGL2331)]

Purified cell walls were hydrolysed with acid and the resulting materials were derivatized and analysed by GC, as described in Methods. The values are the means ± SD of at least three independent experiments.

Strain	Ara : Gal	Rha : Gal
Wild-type	4.50 ± 0.5	1.0 ± 0.1
Cg-Δ186	2.20 ± 0.2	0.56 ± 0.05
Cg-Δ186(pCGL2330)	4.60 ± 0.5	0.60 ± 0.05
Cg-Δ186(pCGL2331)	4.30 ± 0.3	0.60 ± 0.05

In vitro epimerization of DPR into DPA by cell membrane and cell wall from Cg-Δ186 and wild-type strains

To prove the direct involvement of *NCgl0186* in DPA biosynthesis in *C. glutamicum* we determined the effect of mutation of the coding gene on the epimerization of DPR into DPA. Thus, both membranes and cell walls prepared from Cg-Δ186 and its parental strain, ATCC 13032, were independently incubated with radioactive p[¹⁴C]Rpp and decaprenyl phosphate. The resulting mixtures of DP[¹⁴C]R and DP[¹⁴C]A were hydrolysed and the radioactive sugars released were analysed by HPLC. The epimerization of DPR into DPA was observed when wild-type cell membranes and, to a lesser extent, wild-type cell walls were used as enzyme sources (Table 2). When the experiments were done with Cg-Δ186 cell walls and cell membranes we observed a 50% decrease in the DPA formed as compared with the wild-type. This change was partially restored by complementation with *NCgl0186*. Overexpression of *NCgl0186* in the wild-type bacterium did not lead to increased epimerization (Table 2), probably due to the fact that *NCgl0186* was not limiting for epimerization in the wild-type bacterium. These data established the direct involvement of *NCgl0186* in the epimerization of DPR into DPA and confirmed the contribution of another enzyme with similar function.

Structural analysis of the AG from the cell walls of Cg-Δ186 and wild-type strains

In order to investigate the potential effects of *NCgl0186* deletion on the structure of AG we performed different types of analysis. First, the soluble AG from Cg-Δ186 and wild-type were treated with an arabinase partially purified from *Mycobacterium smegmatis* (Dong *et al.*, 2006), and the reaction products were analysed by MALDI-TOF MS. Similar fragments were observed in the degradative products of the AG from the wild-type and Cg-Δ186 strains; they were composed of oligomers of Ara of up to nine residues. Second, the soluble AGs from both strains were treated with a mixture of arabinases and galactofuranases from *Cellulomonas* (McNeil *et al.*, 1994), and analysed by HPLC and MALDI-TOF MS. Again, no significant difference could be detected between the patterns obtained from Cg-Δ186 and ATCC 13032 strains by this method (data not shown). Consistent with these observations, the glycosyl-linkage compositions of the AG from the wild-type and mutant strains of *C. glutamicum* were very similar to one another and to those of other strains of the species (Alderwick *et al.*, 2005; Gebhardt *et al.*, 2007). Finally, since ¹³C-NMR spectroscopy of AGs has proven to be an appropriate tool for comparative qualitative analysis of AGs, AG from the two isogenic strains was compared by this technique (Daffé *et al.*, 1990, 1993; Gebhardt *et al.*, 2007). The ¹³C-NMR spectra obtained from Cg-Δ186 and ATCC 13032 were superimposable (data not shown) and identical to that published

previously for another strain of *C. glutamicum* (Gebhardt *et al.*, 2007). We thus concluded that the AGs from the two strains were composed of the same structural motifs.

Lipid analysis of the Cg-Δ186 and wild-type strains

In view of the decrease in the Ara content of the AG from Cg-Δ186, we reasoned that this might affect the lipid profile of the mutant, notably the amount of corynomycolates linked to AG. Accordingly, we performed a comparative lipid analysis of Cg-Δ186 and the wild-type strain, focusing on corynomycolate-containing compounds, i.e. cell wall and trehalose monocorynomycolate (TMC) and trehalose dicorynomycolate (TDCM) (Puech *et al.*, 2001). Cells were labelled with acetate and the lipids were extracted with organic solvents. Analysis of the extractable lipids by TLC and quantification of the corynomycolate esterifying trehalose showed that the mutant strain accumulated 57 % more corynomycolates than did the parent strain (Table 3). Concomitantly, the Cg-Δ186 strain contained 40 % less corynomycolates covalently bound to AG (Table 3). These data showed that *NCgl0186* deletion affected the tethering of corynomycolates to AG, probably through the reduction of the number of sites of attachment of these lipids. Consistent with this finding, this phenotype was completely reversed in the mutant strain complemented with either pCGL2330 (containing *NCgl0186*) or pCGL2331 (containing *Rv3191*). We thus concluded that Cg-Δ186 elaborated an AG with fewer Ara units and corynomycoloyl residues but with structural motifs qualitatively similar to those of the parental strain.

Table 2. Epimerization of DPR to DPA by cell membranes and cell walls of *C. glutamicum* ATCC 13032, its isogenic Δ*NCgl0186* (Cg-Δ186) mutant and the complemented mutant Cg-Δ186(pCGL2330), and the wild-type strain in which *NCgl0186* was overexpressed [ATCC 13032(pCGL2330)]

Epimerase activity was assayed by measuring the ability of cell wall or cell membrane extracts to transform pRpp into DPA. The percentages of Ara and ribose were determined relative to the sum of the two sugars present in the isolated mixture. The values are the means $\pm$ SD of at least three independent experiments.

Strain	Cell wall as enzyme source		Cell membrane as enzyme source	
	Ara (%)	Ribose (%)	Ara (%)	Ribose (%)
ATCC 13032	72 $\pm$ 4	28 $\pm$ 4	83 $\pm$ 2	17 $\pm$ 3
Cg-Δ186	41 $\pm$ 3	59 $\pm$ 3	51 $\pm$ 3	49 $\pm$ 3
Cg-Δ186(pCGL2330)	44 $\pm$ 3	56 $\pm$ 3	63 $\pm$ 4	37 $\pm$ 4
ATCC 13032(pCGL2330)	62 $\pm$ 2	38 $\pm$ 2	78 $\pm$ 3	22 $\pm$ 3

Table 3. Quantitative analysis of cell wall-linked and extractable corynomycolates from *C. glutamicum* ATCC 13032, its isogenic mutant Δ*NCgl0186* (Cg-Δ186) and the mutant complemented with either the corynebacterial gene [Cg-Δ186(pCGL2330)] or its mycobacterial orthologue [Cg-Δ186(pCGL2331)]

The percentages of corynomycolates in cell walls and extractable lipids were determined relative to the masses of the delipidated cells and extractable lipids, respectively. The values are the means $\pm$ SD of at least three independent experiments. Corynomycolates were isolated by saponification of the delipidated cells and extractable lipids, purified by chromatography and weighed, as described in Methods.

Strain	Wall-linked corynomycolates (%)	Corynomycolates in extractable lipids (%)
ATCC 13032	1.0 $\pm$ 0.1	33 $\pm$ 2
Cg-Δ186	0.6 $\pm$ 0.1	52 $\pm$ 3
Cg-Δ186(pCGL2330)	1.1 $\pm$ 0.1	29 $\pm$ 2
Cg-Δ186(pCGL2331)	1.0 $\pm$ 0.1	28 $\pm$ 2

Search for an NCgl0186 orthologue and construction of the corresponding mutant

As DPA has been shown to be the only donor of Ara in *C. glutamicum* (Alderwick *et al.*, 2005), these results indicated that DPA is still produced by the Cg-Δ186 mutant, as confirmed by enzyme assay (Table 2). As a consequence, the occurrence of Ara in the mutant could be explained by the redundancy of *NCgl0186* in *C. glutamicum* or, alternatively, *NCgl0186* could have a function in corynebacteria different from that of *Rv3791* in mycobacteria, despite the homology and the similar genomic environment of the two genes, i.e. *Rv3791* and *NCgl0186*. However the last hypothesis seems unlikely in view of our data that show that the expression of *Rv3791* is able to complement the deficit of Cg-Δ186 in Ara. In order to identify genes encoding one or more proteins functionally equivalent to *NCgl0186* in *C. glutamicum*, BLAST searches were performed against this genome using *NCgl0186* as a query sequence (Altschul *et al.*, 1990). On the basis of the score values, only one sequence, the product of the *NCgl1429* gene, could be considered to encode a paralogous protein. This protein shows 35 % identity with *NCgl0186* (Fig. 3). We checked for the occurrence of an *NCgl1429* orthologue in the *M. tuberculosis* genome and found *Rv2073c*. As shown in Fig. 3 amino acid alignments of *NCgl1429* and *Rv2073c* with *NCgl0186* and *Rv3791* revealed well-conserved blocks, and led us to propose *NCgl1429* as the protein possessing a functional redundancy with *NCgl0186*. It should be noted that, in contrast to *NCgl0186*, no paralogue was found for *NCgl0187* in *C. glutamicum*. We thus attempted to delete *NCgl1429* both in ATCC 13032 and in Cg-Δ186.

A *km* resistance cassette flanked by arms about 500 bp identical to the 5' and 3' parts of *NCgl1429* was cloned into

Fig. 3. Multiple alignments of protein sequences of NCgl0186, Rv3791, NCgl1429, Rv2073c and the deduced consensus sequence (consensus 1). Identical residues are boxed and similar amino acids have light-grey backgrounds. The line above the sequence indicates the conserved block chosen for computational analysis. The first three secondary elements predicted from the NCgl0186 sequence are shown. Consensus 2 corresponds to the consensus sequence obtained from CLUSTAL W alignments of all the orthologues found in *Corynebacterineae* given in Supplementary Table S2, except for MAP1819c of *M. paratuberculosis*, which was not considered for the end of the alignment owing to its length (187 residues). Boxed residues in consensus 2 correspond to a conserved motif that may be involved in cofactor binding.

a vector unable to replicate in corynebacteria. This construct was transferred into both *C. glutamicum* ATCC 13032 and Cg- Δ 186. In both cases *km^R* transformants were obtained and analysed by PCR using various combinations of primers. From the wild-type strain one clone (Cg- Δ 1429) among 20 gave the amplification pattern consistent with allelic replacement of NCgl1429 with the *km* cassette. This exchange was the result of two recombination events. In the Cg- Δ 186 host only the first recombination event, corresponding to plasmid integration, could be obtained, as revealed by the analysis of 200 clones. This absence of deletions led us to suspect that, as for NCgl0187, inactivation of both genes (i.e. NCgl0186 and NCgl1429) was not possible because of the essentiality of the function associated with the two proteins in the epimerization of DPR to DPA. To conclusively demonstrate this hypothesis we tried to inactivate NCgl1429 in Cg- Δ 186 by plasmid integration in the presence or absence of a functional copy of NCgl0186. For that purpose a pCR2.1-TOPO plasmid derivative containing an ~500 bp NCgl1429 internal fragment was first constructed (TOPO::1429). We then transferred a copy of NCgl0186 under the control of the

cspB promoter into this plasmid (TOPO::1429-cspB186). No integration mutants could be obtained when Cg- Δ 186 was transformed with TOPO::1429, while integration took place very easily when TOPO::1429-cspB186 was used as integration vector. These data demonstrated that NCgl1429 is essential in the absence of NCgl0186.

Analysis of the NCgl1429 mutant strain

Cell walls from Cg- Δ 1429 were analysed for glycosyl composition and corynomycolate content as described for Cg- Δ 186 and ATCC 13032. Surprisingly, and in sharp contrast with Cg- Δ 186, no change in the Ara:Gal and Rha:Gal ratios could be detected in the mutant strain, compared with the wild-type. Likewise, no significant difference was observed in terms of amounts of extractable and bound corynomycolates of the mutant, compared with the parental ATCC 13032 strain. These results strongly suggest that in the presence of a functional NCgl0186, NCgl1429 has no detectable contribution *in vivo*. Based on the data obtained previously with the partially redundant mycolyltransferases of *M. tuberculosis* (Puech *et al.*, 2002),

we tested the *in vivo* effect of *NCgl1429* overexpression on the epimerization reaction in the absence of *NCgl0186*. For that purpose, a plasmid containing the *NCgl1429* ORF under the control of the strong promoter of *cspB* was constructed (pCGL2333) and introduced into the Cg- Δ 186 strain. As we have shown that the quantification of corynomycolates linked to AG reasonably reflects the complementation of the phenotype of the Cg- Δ 186 mutant (Table 3), we performed a quantitative analysis of the cell wall corynomycolates of Cg- Δ 186(pCGL2333). The percentage of cell wall-linked corynomycolates of Cg- Δ 186(pCGL2333) was found to be 1% (± 0.1), a value close to that obtained with the wild-type strain and with the Cg- Δ 186 strain complemented with either *NCgl0186* or *Rv3791* (Table 3). This result indicated that overexpression of *NCgl1429* *in vivo* could restore the defect in AG produced by the absence of *NCgl0186*, thus pointing to a partial redundancy of the two enzymes similar to that demonstrated for the three mycolyltransferases of *M. tuberculosis* (Puech *et al.*, 2002).

Conservation and redundancy of *NCgl0186* orthologous proteins among *Corynebacterineae*

NCgl0186, *NCgl1429*, *Rv3791* and *Rv2073c* belong to the superfamily of short chain dehydrogenases/reductases (SDRs), which contains enzymes catalysing NAD(P)(H)-dependent oxido-reduction (Jornvall *et al.*, 1995; Oppermann *et al.*, 2003). This is a large and functionally heterogeneous protein family with low sequence identities but with conserved motifs and common structural organization. In bacteria the number of SDR members deduced from genome analysis varies considerably from one organism to another, but it should be noted that this number is especially large in *M. tuberculosis* (57 members) (Jornvall *et al.*, 1999). We wanted to evaluate the level of sequence conservation and of redundancy of *NCgl0186* orthologues among the *Corynebacterineae*. For this purpose, one of the conserved blocks, SSxAGxRxRRxNx-VYGSxKAGD (shown in Fig. 3), was chosen. It contains the three conserved residues of the catalytic active triad (S, Y and K) characteristic of SDR proteins (Oppermann *et al.*, 2003). This motif was searched for in the NCBI Reference Sequence (RefSeq) collection (<http://www.ncbi.nlm.nih.gov/RefSeq/>). Forty-seven sequences were found to match

with this motif totally and six more with one mismatch. Pair-wise alignments showed that 52 of the 53 sequences possessed between 36 and 100% identities with *Rv3791* and thus can be considered to be closely related. All these protein sequences belong to *Actinomycetales*, mostly to *Corynebacterineae* (see Supplementary Table S2). This analysis shows that all members of *Corynebacterineae* for which the genome sequence is known possess at least one, and the great majority two, orthologous genes.

The SDR superfamily is divided into five families on the basis of protein length and specific sequence motifs. According to their length (between 242 and 260 aa, except for MAP1819c from *Mycobacterium paratuberculosis*, which has only 187 residues) the proteins identified in this work are classical SDRs. CLUSTAL W alignment (Fig. 3) and secondary structure predictions performed on these protein sequences allowed the identification of only two SDR motifs specific to the classical family among the seven proposed by Kallberg *et al.* (2002) (Table 4). In particular, the typical pattern of three Gly residues (TGxxxGxG) in the N-terminal domain characteristic of the NAD(P)(H)-binding site is missing. Nevertheless, a conserved motif, GG/AxSxxG/A, localized in the β 1- α 1 predicted structure that resembles the patterns of both intermediate SDRs (G/AxxGxxG/A) and divergent SDRs (GxxxxxSxA), could constitute an atypical dinucleotide-binding motif (Fig. 3, Table 4).

DISCUSSION

The mAGP complex, which constitutes the cell wall skeleton of the very singular cell envelope of *Corynebacterineae*, represents, with mycolic acids, the hallmark of all the members of this group of bacteria (Besra *et al.*, 1995; Daffé, 2005; Dover *et al.*, 2004; McNeil *et al.*, 1991). Despite the physiological importance of this complex, its biosynthesis is far from being totally understood, mostly because of its essentiality for the viability of *M. tuberculosis*. However, thanks to the use of *C. glutamicum* as a model organism for *Corynebacterineae*, significant progress on understanding AG biosynthesis has been made recently, in particular with the discovery of new glycosyltransferases (Alderwick *et al.*, 2006b; Seidel *et al.*, 2007). As AG is made up of D-arabinofuranosyl and

Table 4. Conserved motifs in the *NCgl0186* orthologue group and comparison with classical SDR motifs

Secondary structure elements were predicted using PredictProtein (Rost *et al.*, 2004). In the motif, 'a' denotes an aromatic residue, 'h' a hydrophobic residue and 'x' any residue. Alternative amino acids at a motif position are given within brackets.

Secondary structure element	Classical SDR motif*	<i>NCgl0186</i> orthologue group motif	Suggested function*
β 1 + α 1	TGxxGhaG	G[GA]xSx[EDQ][IM][GA]	Structural role in cofactor-binding region
β 3 + α 3	DAx[DV]	[DEN]Ax[DV]	Binding of coenzyme
α 5	Yx[AS][ST]K	YGS[AT]K	Part of active site

*Persson *et al.* (2003).

D-galactofuranosyl units, which are both rare sugars (McNeil *et al.*, 1987), a complete understanding of their biosynthetic pathways could provide suitable targets for new chemotherapeutics. Ara is produced from DPA, the formation of which in mycobacteria has been shown to proceed by an unusual epimerization reaction that occurs when the sugar is linked to a prenyl phosphate (Mikusova *et al.*, 2005). Previous experiments performed with purified recombinant Rv3790 and Rv3791 proteins from *M. tuberculosis* have shown that DPR could be converted into DPA in an *in vitro* system when both proteins were present in the reaction mixtures (Mikusova *et al.*, 2005). Despite the fact that Rv3790 and Rv3791 were sufficient to catalyse the epimerization of DPR *in vitro*, there is no indication that, in a physiological context, other proteins are not also involved in this reaction. To investigate the *in vivo* functions of these proteins in the biosynthesis of AG, we deliberately chose to inactivate their orthologues *NCgl0186* and *NCgl0187* in *C. glutamicum*. This strategy was based on the fact that, unlike mycobacteria, corynebacteria can survive with a truncated AG totally devoid of the arabinan domain (Alderwick *et al.*, 2005), and because of the synteny observed for the 3790/3791 locus from genome analysis of numerous *Corynebacterineae*, suggesting conserved functions for the orthologous proteins.

While the deletion of *NCgl0186* was very easy to achieve, all our attempts to delete *NCgl0187* were unsuccessful, probably reflecting the essentiality of this gene. The earlier generation of a *ubiA* mutant in *C. glutamicum* and its analysis has shown that DPA is the only Ara donor for AG biosynthesis and, as a consequence, its total absence leads to the production of a galactan instead of an AG (Alderwick *et al.*, 2005, 2006a). Cg-Δ186 showed a different pattern: the strain possesses an AG with the same structural motifs as the wild-type strain but contains a significantly reduced quantity of arabinosyl residues. The fact that arabinan was reduced but not totally absent demonstrated a role for *NCgl0186* in DPA synthesis, but either in a way different from that observed for Rv3791 *in vitro* or with functional redundancy in *C. glutamicum*. The first hypothesis is unlikely for two reasons. First, a direct involvement of *NCgl0186* in DPR epimerization was verified by *in vitro* experiments. Second, a normal level of Ara was restored by *in vivo* Rv3791 expression in Cg-Δ186. An *in silico* search for paralogues of *NCgl0186* revealed only one gene, *NCgl1429*, which is conserved among all *Corynebacterineae* except *M. leprae* and *Mycobacterium bovis* species. Although the genetic organization around *NCgl1429* or its orthologues differs from one species to another, the gene of interest is always in close proximity, and likely in some cases in an operon together with genes encoding proteins predicted to be involved in precorrin biosynthesis. As in the case of *NCgl0186*, deletion of *NCgl1429* was very easy to realize in the wild-type strain but, in contrast to Cg-Δ186, the resulting mutant synthesized an AG with an Ara:Gal ratio identical to that of the wild-type strain. However, the

hypothesis that *NCgl1429* is involved in DPA synthesis is strongly supported by the following observations. (i) *NCgl1429* could be inactivated in the presence of a copy of *NCgl0186* but not in its absence, indicating that *NCgl1429* becomes essential when *NCgl0186* is absent. (ii) A total recovery of cell wall mycolates was obtained when *NCgl1429* was overexpressed in the absence of *NCgl0186*, indicating that *NCgl1429* could fully replace *NCgl0186* in the epimerization reaction under these conditions. We thus propose that epimerization of DPR into DPA involves three enzymes that catalyse two distinct steps, each being essential for the viability of the cell. One step would be catalysed by *NCgl0187* and another by *NCgl0186*; in the absence of the latter enzyme, the reaction would be catalysed by *NCgl1429*. Although it has been shown previously that epimerization results from DPR oxidation followed by reduction to DPA (Mikusova *et al.*, 2005), we were not able to dissect the two steps by identifying which enzyme is responsible of the oxidation reaction and which catalyses the reduction reaction.

By deleting *NCgl0186* we obtained, for what is believed to be the first time, a mutant in which the DPA synthesis is lowered, allowing us to determine the influence of DPA level on AG biosynthesis *in vivo*. Considering the significant diminution of Rha and corynomycolates observed for the Cg-Δ186 mutant and the occurrence of similar structural motifs in the mutant and the wild-type in our structural analyses of AG, it seems that a decrease in DPA synthesis leads to a decrease in the number of branching arabinan chains rather than in the length of these chains. These results suggest that changes in the level of DPA could influence the number of priming sites for AraF deposition to the galactan core but not (or to a lesser extent) Ara polymerization.

The essentiality of the epimerization step was unexpected as the inactivation of the gene encoding the first enzyme involved in DPA synthesis, the DPPR synthase (*UbiA*), in *C. glutamicum*, produced a viable mutant (Alderwick *et al.*, 2005). One possible reason is that absence of epimerization leads to a continued synthesis of DPR, which in turn significantly affects the pool of decaprenyl phosphate, so that this becomes limiting for peptidoglycan synthesis and lethal for the cell. This hypothesis has to be proven experimentally.

Apparent redundancy of gene function appears to be common among genes involved in a wide range of physiological processes. This redundancy has been found for some enzymes involved in cell wall biosynthesis in *Corynebacterineae*, such as mycoloyltransferases in corynebacteria (Brand *et al.*, 2003; De Sousa-D'Auria *et al.*, 2003) and mycobacteria (Puech *et al.*, 2002), and Embs in mycobacteria (Escuyer *et al.*, 2001). It could also be the case for the enzyme that dephosphorylates DPPR to DPR. This phosphatase is believed to be encoded by the gene located directly upstream of *ubiA* (*Rv3807c* in *M. tuberculosis* and *NCgl2782* in *C. glutamicum*). Preliminary

experiments with an *NCgl2782* mutant of *C. glutamicum* indicate that if this gene is effectively the phosphatase, a redundant enzyme must exist, as in the case of *NCgl0186*. In agreement with this, *in silico* analysis showed a paralogue (*NCgl2785*) located very close to *NCgl2782*. Further experiments will be needed to determine the precise role of the enzymic redundancies observed in the synthesis of DPA and more generally in cell wall compound biosynthesis.

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