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Carbohydrate Metabolism Differences between Subgroup A1 and B2 Strains of *Bacillus anthracis* as Assessed by Comparative Genomics and Functional Genetics▽

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In France, *Bacillus anthracis* subgroup B2 strains do not metabolize starch or glycogen but can use gluconate, whereas subgroup A1 strains show the inverse pattern. Functional genetic analysis revealed that mutations in the *amyS* and *gntK* genes encoding an alpha-amylase and a gluconate kinase, respectively, were responsible for these phenotypes.

Bacillus anthracis, the etiological agent of anthrax, is a gram-positive, aerobic soil bacterium. Multilocus variable-number tandem repeat analysis of a collection of French isolates shows that the main groups of *B. anthracis* groups A (subgroup A1) and B (subgroup B2) described worldwide are represented (1, 2). Subgroup B2 isolates are the most common isolates in France and are found particularly in southern mountain regions, but they are extremely rare elsewhere in the world. Biochemical characterization of French isolates indicates that subgroup A1 and B2 strains have different carbohydrate utilization patterns (P. Vaissaire, A. Fouet, K. L. Smith, C. Keys, C. Le Doujet, P. Sylvestre, M. Levy, P. Keim, and M. Mock, presented at the 5th International Conference on Anthrax and 3rd International Workshop on the Molecular Biology of *Bacillus cereus*, *B. anthracis* and *B. thuringiensis*, 30 March to 3 April 2003, Nice, France). French subgroup A1 strains metabolize starch and glycogen but not gluconate, and the inverse is true for subgroup B2 strains. The genomes of several *B. anthracis* strains are available on the NCBI website (http://www.ncbi.nlm.nih.gov/), and two of these strains, Ames and CNEVA, are representative of groups A and B, respectively. We compared the genomic sequences of Ames and CNEVA to identify mutations that may affect metabolic activities involved in the phenotypic differences.

The Kegg pathway database (http://www.genome.jp/kegg/pathway.html) was used to select enzyme activities involved in the metabolic pathways for starch, glycogen, and gluconate. BLAST analysis of the corresponding open reading frame in the Ames (subgroup A3) and CNEVA (subgroup B2) genomes was then used to identify the selected genes that were interrupted or mutated. The functions and localizations of these open reading frames were then investigated with the Pfam (http://pfam.sanger.ac.uk/), CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml), SMART (http://smart.embl-heidelberg.de/), SignalP (http://www.cbs.dtu.dk/services/SignalP/), and TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) search programs. A single-base deletion in the *amyS* gene (BA3551) encoding an alpha-amylase linked to starch and glycogen metabolism was found in the CNEVA genome. The wild-type AmyS protein contains 513 amino acids, and its predicted molecular mass is 58.4 kDa. In subgroup B2, there is a frameshift due to deletion of an adenosine in the 7th position of the nucleotide sequence that leads to a premature stop codon in the 13th position. In the Ames genome, a single-base substitution was found in the *gntK* gene (BA0162) encoding a gluconate kinase linked to gluconate metabolism. The predicted wild-type GntK protein contains 511 amino acids, and its predicted molecular mass is 56.7 kDa. The mutation identified is a cytosine-to-adenosine substitution at position 530 of the nucleotide sequence that leads to a premature stop codon at amino acid position 176. We confirmed the presence of these two mutations in the other *B. anthracis* subgroup genomes accessible in the NCBI unfinished microbial genome database and sequenced 12 isolates with various genotypes belonging to subgroups A1 and B2 (6 isolates in each subgroup) originating from outbreaks that occurred in different regions of France over the last 15 years. These analyses revealed that the deletion in *amyS* is restricted to strains belonging to group B subgroups, whereas the substitution in *gntK* is restricted to strains belonging to group A subgroups. The mutations identified in *amyS* and *gntK* both result in premature stop codons that lead to a loss of the enzymatic activities and may thus account for the observed phenotypic differences between subgroup A1 and B2 strains. We therefore focused on these two genes and used French strains 9602R and RA3R belonging to subgroups A1 and B2, respectively, for further analysis.

Phenotypic analysis of *amyS* and *gntK* mutants and derivatives. We constructed *amyS* and *gntK* deletion mutants and complemented strains to determine the involvement of the amylase AmyS and of the gluconate kinase GntK in starch-glycogen and gluconate utilization. Genetic construction was performed as previously described (5, 6). The conjugative suicide pAT113 (8) and replicative pAT187 (7) recombinant plas-

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TABLE 1. Starch, glycogen, and gluconate phenotypes of *B. anthracis* subgroup A1 and B2 strains and derivatives used in this study

Strain	Genotype or description	Use of:			Marker(s)[a]	Reference
		Starch	Glycogen	Gluconate		
9602R	pXO1$^+$, subgroup A1	+	+	−		6
RA3R	pXO1$^+$, subgroup B2	−	−	+		6
PF106	9602R Δ*amyS*	−	−	−	Sp^r	This study
PF114	9602R Δ*amyS*(pAT187-*amyS*$_{9602R}$)	+	+	−	Sp^r Kn^r	This study
PF115	RA3R(pAT187-*amyS*$_{9602R}$)	+	+	+	Kn^r	This study
PF128	RA3R nonpolar Δ*gntK*	−	−	−	Sp^r	This study
PF147	RA3R nonpolar Δ*gntK*(pAT187-*gntRK*$_{RA3R}$)	−	−	+	Sp^r Kn^r	This study
PF152	9602R(pAT187-*gntRK*$_{RA3R}$)	+	+	+	Kn^r	This study

[a] Kn^r, resistance to kanamycin; Sp^r, resistance to spectinomycin.

mids were introduced into *B. anthracis* by "heterogramic" conjugation (i.e., between gram-negative and gram-positive bacteria) (7). Gene deletion was performed by allelic exchange of the wild-type copy with a copy interrupted by an antibiotic resistance cassette. The abilities of the strains to grow with starch, glycogen, and gluconate as carbon sources were tested with the API 50CH standardized system from Biomérieux used according to the manufacturer's instructions (Table 1).

The *amyS* gene from subgroup A1 strain 9602R was interrupted by a polar spectinomycin resistance cassette (4). The resulting *amyS* mutant (PF106) failed to use either starch or glycogen (Table 1). The complemented strain, PF114 harboring the replicative plasmid pAT187 carrying the *amyS* gene under its own promoter, was able to use starch and glycogen (Table 1). Strain RA3R, in which the *amyS* gene is naturally mutated, was complemented with the same recombinant plasmid; the resulting strain (PF115) was also able to use starch and glycogen (Table 1).

The *B. anthracis gntK* gene belongs to a cluster of four open reading frames (Fig. 1). To test the involvement of the *gntK* gene in *B. anthracis* gluconate metabolism, a deletion mutant strain, PF128 containing the *gntK* gene interrupted by a nonpolar spectinomycin resistance cassette (3), was constructed using the parental subgroup B2 strain RA3R. This mutant did not use gluconate as a sole carbon source (Table 1). For complementation, a conjugative replicative plasmid carrying the *gntR* and *gntK* genes and the *gntR* promoter region was constructed, because the overlapping stop and start codons (8 nucleotides) of these two genes suggest that they may be co-

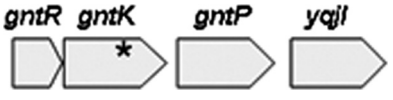

FIG. 1. Gluconate gene cluster organization. The *gntR* (BA0161), *gntK* (BA0162), *gntP* (BA0163), and *yqjI* (BA0164) genes encode a potential gluconate operon transcriptional regulator, a gluconate kinase, a gluconate permease, and a 6-phosphogluconate dehydrogenase, respectively. The *gntR* and *gntK* genes have overlapping stop and start codons (8 nucleotides), and the intergenic regions between *gntK* and *gntP* and between *gntP* and *yqjI* are 119 and 264 nucleotides long, respectively.

transcribed. Mutant strain PF128 complemented with this plasmid, pAT187-gntRK$_{RA3R}$, was able to use gluconate (PF147) (Table 1). The same recombinant plasmid was introduced into strain 9602R, in which the *gntK* gene is naturally mutated; the complemented strain, PF152, was also able to use gluconate.

In conclusion, we show here that the mutations identified in the *amyS* and *gntK* genes are directly responsible for the phenotypic differences in starch, glycogen, and gluconate degradation between subgroup A1 and B2 strains. We previously reported that the glycosylation patterns of BclA, a structural component of the exosporium, differ in group A and subgroup B2 strains (6). Here, we provide a second example of phenotypic variations that could be used to differentiate strains. Consequently, sequencing the *amyS* and *gntK* genes may allow rapid discrimination between the major *B. anthracis* subgroup lineages, and a more complete phylogenetic study using multilocus variable-number tandem repeat analysis is required only for confirmation.

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