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Chromosomal *aadD2* Encodes an Aminoglycoside Nucleotidyltransferase in *Bacillus clausii*

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***Bacillus clausii* SIN is one of the four strains of *B. clausii* composing a probiotic administered to humans for the prevention of gastrointestinal side effects due to oral antibiotic therapy. The strain is resistant to kanamycin, tobramycin, and amikacin. A gene conferring aminoglycoside resistance was cloned into *Escherichia coli* and sequenced. The gene, called *aadD2*, encoding a putative 246-amino acid protein, shared 47% identity with *ant(4′)-Ia* from *Staphylococcus aureus*, which encodes an aminoglycoside 4′-*O*-nucleotidyltransferase. Phosphocellulose paper-binding assays indicated that the gene product was responsible for nucleotidylation of kanamycin, tobramycin, and amikacin. The *aadD2* gene was detected by DNA-DNA hybridization in the three other strains of the probiotic mixture and in the reference strain *B. clausii* DSM8716, although it did not confer resistance in these strains. Mutations in the sequence of the putative promoter for *aadD2* from *B. clausii* SIN resulted in higher identity with consensus promoter sequences and may account for aminoglycoside resistance in that strain. The *aadD2* gene was chromosomally located in all strains and was not transferable by conjugation. These data indicate that chromosomal *aadD2* is specific to *B. clausii*.**

Probiotics are administered to humans for the prevention of gastrointestinal side effects due to oral antibiotic therapy. Ingestion of high quantities of spores is thought to restore an intestinal flora following the destruction of the normal flora by antibiotics (17). Enterogermina is a preparation of *Bacillus* available in Italy (3, 18). It is composed of four antibiotic-resistant strains, OC, NR, T, and SIN (3), initially identified as *Bacillus subtilis* but recently assigned to the *Bacillus clausii* species (26). For the prevention of intestinal disorders, the strains of *Bacillus* used in the Enterogermina preparation were rendered multiantibiotic resistant to survive in the presence of the antibiotics which are coadministered (3, 18). One of the strains, *B. clausii* SIN, is resistant to kanamycin, tobramycin, and amikacin (3). Resistance to aminoglycosides is generally due to enzymatic modification of the drugs and is widespread in a variety of bacterial pathogens (27). However, aminoglycoside inactivation has rarely been reported for *Bacillus* spp. An *aph(3′)-IVa* gene from a butirosin-producing strain of *Bacillus circulans* was reported and subsequently sequenced (4, 10). This gene encodes an aminoglycoside 3′-*O*-phosphotransferase type IV which confers resistance to butirosin, gentamicin B, kanamycin, lividomycin, neomycin, paromomycin, and ribostamycin by modifying these aminoglycosides at the 3′-hydroxyl group (10). *B. subtilis* 168 possesses a chromosomal *aadK* gene which encodes an aminoglycoside 6-*O*-adenylyltransferase that confers resistance to streptomycin (23). A kanamycin 4′-*O*-nucleotidyltransferase encoded by plasmid pTB913 from a thermophilic *Bacillus* sp. was found to be similar to that encoded by the gene *ant(4′)-Ia* or *aadD* borne by plasmid pUB110 from *Staphylococcus aureus* (16). The aim of this study was to characterize the mechanism of resistance of *B. clausii* SIN to aminoglycosides and to elucidate the genetic basis of this mechanism.

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MATERIALS AND METHODS

Bacterial strains. The four *B. clausii* strains OC, NR, SIN, and T used for the production of Enterogermina were obtained from Sanofi-Synthelabo OTC SpA (Milan, Italy) as separate spore suspensions. *B. clausii* DSM8716 was used as a reference strain.

Susceptibility to antibiotics. Antibiotic susceptibility was tested by the disk diffusion method, and MICs were determined by agar dilution according to the recommendations of the National Committee for Clinical Laboratory Standards (21). Susceptible, intermediate, and resistant categories were defined according to the recommendations of the National Committee for Clinical Laboratory Standards (22).

Mating experiments. *Enterococcus faecalis* JH2-2 (11) and *Enterococcus faecium* HM1070 (1), both resistant to rifampin and fusidic acid, and *B. subtilis* UCN19, resistant to ciprofloxacin, were used as recipients in mating experiments (5, 11). The latter strain was obtained by stepwise selection of a mutant from *B. subtilis* 168 (12) on agar plates containing increasing concentrations of ciprofloxacin. In every transfer experiment, *E. faecalis* BM4110 containing the conjugative plasmid pAMβ1 (14) was used as a control. Agar plates for the selection of transconjugants contained rifampin (50 μg/ml) plus fusidic acid (20 μg/ml) or ciprofloxacin (8 μg/ml) combined with 128 μg of kanamycin per ml for enterococci and 20 μg/ml for *B. subtilis*. All mating experiments were repeated a minimum of three times.

PCR. The deoxynucleotide primers ANT4-1 (5′ TAAATATGGGGATGAT GTTAAGGC 3′) and ANT4-2 (5′ TGGTATGCGTTTTGACACATCCAC 3′), specific for *ant(4′)-Ia*, were used. *S. aureus* BM3002 was used as a control (6).

DNA manipulations. Total and plasmid DNA from *Bacillus* strains was extracted as described previously for enterococci (2). Cloning was carried out by standard techniques (25). Total DNA from *B. clausii* strains was digested with various restriction enzymes, cloned into plasmid pUC18, and introduced by electrotransformation into *Escherichia coli* DH10B (2). Recombinant plasmids were selected on agar plates containing ampicillin (200 μg/ml) and kanamycin (20 μg/ml). A DNA fragment conferring kanamycin resistance was then subcloned into the shuttle plasmid pJIM2246 conferring resistance to chloramphenicol (24) and introduced into *E. faecalis* JH2-2 by electrotransformation (1).

TABLE 1. MICs of aminoglycosides for *B. clausii* strains and transformants

Strain	MIC (μg/ml)				
	Kanamycin	Tobramycin	Amikacin	Gentamicin	Netilmicin
B. clausii DSM8716	0.5	0.5	1	0.5	0.5
B. clausii OC	4	2	1	0.5	<0.5
B. clausii NR	2	1	0.5	0.5	<0.5
B. clausii SIN	>1,000	516	16	0.5	<0.5
B. clausii T	4	4	0.5	0.5	<0.5
E. coli DH10B	0.5	1	1	1	0.25
E. coli DH10B/pUV12	32	64	4	1	<0.5
E. faecalis JH2-2	16	8	64	8	1
E. faecalis JH2-2/pUV13	>1,000	>1,000	128	8	1

Sequencing of DNA was performed on ABI PRISM 377 (Perkin-Elmer Corp., Norwalk, Conn.) with big dye terminator according to the protocol supplied by the manufacturer. Nucleotide and amino acid sequences were analyzed by using the software available online at Pedro's Biomolecular Research Tools website (http://www.up.univ-mrs.fr/~wabim/pedro/research_tools.html) and at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). Putative promoters were identified by using the Neural Network Promoter Prediction software (www.fruitfly.org/seq_tools/). Score predictions of >0.9 were considered to be significant.

Southern hybridization. DNA from *B. clausii* strains was digested with I-*Ceu*I or *Sma*I (New England Biolabs, Beverly, Mass.), separated by pulsed-field gel electrophoresis with a technique used for enterococci (2), transferred onto a nylon membrane, and hybridized to a probe specific for *aadD2* of *B. clausii* and consisting of the entire gene amplified by PCR and labeled with digoxigenin (Boehringer Mannheim). DNA was then hybridized to a probe specific for the 16S rRNA. The 16S rRNA probe was obtained by labeling the DNA fragment amplified from *B. clausii* DSM8716 with deoxynucleotide primers 5′ AACTGG AGGAAGGTGGGGAT 3′ and 5′ AGGAGGTGATCCAACCGCA 3′ (7).

Aminoglycoside-modifying enzyme assays. Bacteria were lysed by ultrasonic disintegration, and the resulting extracts were centrifuged at 100,000 × *g* for 45 min. Aminoglycoside-modifying activity in the supernatant (S100) was tested by the phosphocellulose paper-binding technique with [α-^{32}P]ATP (specific activity, 30 Ci/mmol) as described previously (8). Aminoglycosides (67 μg/ml, i.e., 1.4×10^{-1} mM kanamycin or tobramycin) were incubated with S100 extracts at 30°C for 30 min in TMND buffer (0.06 M Tris-HCl, 0.04 M $MgCl_2$, 0.4 M NH_4Cl, 1.6×10^{-4} M dithiothreitol [pH 7.1]).

Nucleotide sequence accession number. The 1,006-bp fragment containing *aadD2* was submitted to GenBank and assigned accession no. AF539790.

RESULTS AND DISCUSSION

Antibiotic resistance in *B. clausii* strains. By disk diffusion, all *B. clausii* strains were found to be resistant to penicillin G, cefalotin, cefotaxime, erythromycin, and lincomycin. Resistance to aminoglycosides was detected only in *B. clausii* SIN, and the MICs of certain aminoglycosides for the *B. clausii* strains are shown in Table 1. *B. clausii* SIN was resistant to kanamycin, tobramycin, and amikacin but susceptible to gentamicin and netilmicin.

Characterization of the *aadD2* gene from *B. clausii* SIN. An 8-kb *Eco*RI DNA fragment originating from the DNA of *B. clausii* SIN was cloned into plasmid pUC18. The recombinant plasmid, pUV11, conferred resistance to ampicillin and kanamycin. A 2.2-kb *Sau*3A fragment that also conferred resistance to kanamycin was subsequently subcloned into pUC18, generating plasmid pUV12, and sequenced. Analysis of the sequence revealed the presence of an open reading frame of 765 bp or an alternative open reading frame starting 6 bp upstream and putatively encoding 254- or 256-amino-acid proteins. Both ATG start codons were preceded at 9 and 10 bp by ribosome-binding site-like sequences, 5′ GAGATGGAAG 3′ and 5′ CAAAAGGAGA 3′, complementary to five and seven bases (underlined), respectively, of the 3′-OH-terminal sequence (5′ UCUUUCCUCC 3′) of *B. subtilis* 16S rRNA (19).

A search for the presence of motifs in the deduced amino acid sequence detected a nucleotidyltransferase domain between amino acids 14 and 59 that is shared by a large family of nucleotidyltransferases (15). In this family, highest homology (identity, 47%; similarity, 65%) was found with the enzyme encoded by *ant(4′)-Ia* or *aadD* (GenBank accession no. V01282) of plasmid pUB110 from *S. aureus* (Fig. 1) (20, 27). As previously mentioned, a gene that is identical, except for a 1-bp

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SIN          (MN)MNGPASMAQKERLQTCQEIAKRLHEVYGNDVLAIGVYGSVSRGTDGPFSDIEMFCVLRDS
pUB110           MNGPIIMTREERMKIVHEIKERILDKYGDDVKAIGVYGSLGRQTDGPYSDIEMMCVMST-
                 ****  *   **     **  *    ** ** *******  * **** ***** **

SIN              AETVDKSYEWSAGPWKAEVNVCSASILLKDAATVEDRWPLTHGPYFSPLRLYDPEGFFQR
pUB110           -EEAEFSHEWTTGEWKVEVNFDSEEILLDYASQVESDWPLTHGQFFSILPIYDSGGYLEK
                  *    * **  * ** ***  *  ***  *  **  ******  ** *  **  *

SIN              LRLAAESPTKEDFRQAIHEILVGEMYEYVGKLRNVNRNGPSTYLPSLALRFAHYGAMLIG
pUB110           VYQTAKSVEAQTFHDAICALIVEELFEYAGKWRNIRVQGPTTFLPSLTVQVAMAGAMLIG
                     * *     *  **    * *  ** ** **    ** * ****    *  ******

SIN              LHNQTLFSTGAMVLPEALKLPHRPKGFDHVAELAMSGDLAQPAKIVSACEDFWKGLVAWA
pUB110           LHHRICYTTSASVLTEAVKQSDLPSGYDHLCQFVMSGQLSDSEKLLESLENFWNGIQEWT
                 **      * * ** ** *    * * **     *** *    *     * ** *   *

SIN              AEHDYVIHS-KRIPF
pUB110           ERHGYIVDVSKRIPF
                   * *     *****
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FIG. 1. Amino acid sequence comparison of AadD2 from *B. clausii* SIN with Ant(4′)-Ia or AadD encoded by plasmid pUB110 from *S. aureus* (16). Sequence identity is indicated by asterisks.

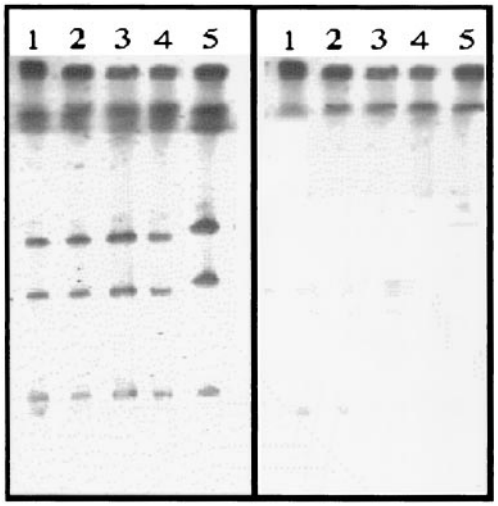

FIG. 2. Localization of *aadD2* in *B. clausii*. Total DNA from *B. clausii* strains NR (lanes 1), OC (lanes 2), T (lanes 3), SIN (lanes 4), and DSM8716 (lanes 5) was digested with restriction enzyme I-*Ceu*I and subjected to pulsed-field gel electrophoresis. DNA was transferred to a nylon membrane and hybridized successively with *rrs* (16S rRNA) (left) and *aadD2* (right) probes labeled with digoxigenin. The low-molecular-weight I-*Ceu*I band is barely visible in lanes 2, 3, 4, and 5 (left).

substitution, was reported in a thermophilic *Bacillus* sp., where it was borne by plasmid pTB913 (16). This nucleotidyltransferase modifies kanamycin, tobramycin, and amikacin and confers a resistance phenotype similar to those of *B. clausii* SIN and the *E. coli* transformant (Table 1).

The *aadD2* gene was subcloned into shuttle vector pJIM2246, and the recombinant plasmid, pUV13, was electrotransformed into *E. faecalis* JH2-2. For the transformants, the MICs of kanamycin and tobramycin were elevated and that of amikacin was moderately higher but the MICs of gentamicin and netilmicin remained unchanged. The resistance phenotype was consistent with modification of the 4′-hydroxyl group of aminoglycosides.

Analysis, by the phosphocellulose paper-binding assay, of bacterial extracts from *E. coli* DH10B/pUV12 indicated that aminoglycoside resistance was due to a nucleotidylating activity. Kanamycin A nucleotidylation was defined as 100%, and tobramycin (91%), amikacin (16%), isepamicin (14%), and neomycin B (100%) were modified at the indicated percentages. Gentamicin C1 and netilmicin were not substrates for the enzyme. On the basis of the substrate profile, the enzyme encoded by pUV12 was an aminoglycoside 4′-*O*-nucleotidyltransferase. Similar results were obtained with *B. clausii* SIN extracts and, surprisingly, also for *B. clausii* DSM8716, although the enzyme activity was much weaker in the latter strain. According to the nomenclature proposed by Shaw et al. (27), the kanamycin nucleotidyltransferase of *B. clausii* was assigned to the Ant(4′)-I enzyme family and the corresponding new gene was tentatively called *aadD2.*

Distribution and localization of *aadD2*. After digestion with the I-*Ceu*I enzyme, which recognizes a sequence which is specific for rRNA operons (13), total DNA of the *B. clausii* strains yielded apparently six fragments, indicating that this species contains a minimum of six rRNA operons (Fig. 2). Southern experiments showed that all fragments hybridized with an *rrs* probe specific for 16S rRNA. The *aadD2* probe hybridized to a single large chromosomal fragment of each strain. *B. clausii*

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DSM8716     AACAGCTATGACATGATTACGAATTCAAGCGTGCGAAATCGTCACTACCTGAAACAAATG
NR          AACAGCTATGACATGATTACGAATTCAAGCGTGCGAAATCGTCACTACCTGAAACAAATG
OC          AACAGCTATGACATGATTACGAATTCAAGCGTGCGAAATCGTCACTACCTGAAACAAATG
T           AACAGCTATGACATGATTACGAATTCAAGCGTGCGAAATCGTCACTACCTGAAACAAATG
SIN         AACAGCTATGACATGATTACGAATTCAAGCGTGCGAAATCGTCACTACCTGAAACAAATG
            ************************************************************
                                      -35          -10 -35
DSM8716     GCAAACAATTATGTCATTTTCCTTGCCAATGAAATGCCTTTTAGGTAACCTTTTCTTAAT
NR          GCAAACAATTATGTCATTTTCCTTGCCAATGAAATGCCTTTTAGGTAACCTTTTCTTAAT
OC          GCAAACAATTATGTCATTTTCCTTGCCAATGAAATGCCTTTTAGGTAACCTTTTCTTAAT
T           GCAAACAATTATGTCATTTTCCTTGCCAATGAAATGCCTTTTAGGTAACCTTTTCTTAAT
SIN         GCAAACAATTATATCATTTTCCTTGACAATGAAATGCCTTTTTGGTAACCTTTTCTTAAT
            ************ ************ *************** *****************
               -10
DSM8716     CAAACATATAGGGGTGGTTCAAGTGCACCGAAAAAAAGTCCCCCGTTAATCTCGAGGTCC
NR          CAAACATATAGGGGTGGTTCAAGTGCACCGAAAAAAAGTCCCCCGTTAATCTCGAGGTCC
OC          CAAACATATAGGGGTGGTTCAAGTGCACCGAAAAAAAGTCCCCCGTTAATCTCGAGGTCC
T           CAAACATATAGGGGTGGTTCAAGTGCACCGAAAAAAAGTCCCCCGTTAATCTCGAGGTCC
SIN         CAAACATAGGGGTGGTTCAAGTGCACCGAAAAAAAGTCCCCCGTTAATCTCGAGGTCC
            ************************************************************

DSM8716     TCCATTCATCGTTCATGTCACACACTCAAATTGAAACAAAAGGAGATGGAAGTGAATGAATATG
NR          TCCATTCATCGTTCATGTCACACACTCAAATTGAAACAAAAGGAGATGGAAGTGAATGAATATG
T           TCCATTCATCGTTCATGTCACACACTCAAATTGAAACAAAAGGAGATGGAAGTGAATGAATATG
OC          TCCATTCATCGTTCATGTCACACACTCAAATTGAAACAAAAGGAGATGGAAGTGAATGAATATG
SIN         TCCATTCATCGTTCATGTCACACACTCAAATTGAAACAAAAGGAGATGGAAGTGAATGAATATG
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FIG. 3. Sequence comparison of putative promoters for the *aadD2* genes in *B. clausii* NR, OC, SIN, T, and DSM8716. The putative −35 and −10 sequences are indicated in bold. The −35 consensus sequence is TTGACA. A putative transcription start site is indicated by a letter in larger font. Two putative ribosome-binding sites are underlined (single and double lines). Putative start codons are shown in bold italics. Sequence identity is indicated by asterisks.

OC, NR, SIN, and T had indistinguishable *Sma*I-generated patterns, and hybridization showed that the *aadD2*-like genes were borne by a fragment approximately 30 kb in size in the probiotic strains and by a larger fragment of ca. 50 kb in *B. clausii* DSM8716 (data not shown). Thus, *aadD2*-like genes were present in all the strains of *B. clausii* studied but were phenotypically expressed only in *B. clausii* SIN.

Sequence analysis of the 8-kb *Eco*RI insert of plasmid pUV11 revealed the presence, 3′ downstream from the *aadD2* gene, of genes homologous to *blaZ*, *blaI*, and *blaR* from *Staphylococcus*, responsible for the production and regulation of a penicillinase (our unpublished data), a *ytrA*-like gene encoding a putative 135-amino-acid protein homologous to tRNA synthetases, the structural gene for an ABC transporter, and a *gntR*-like gene putatively encoding a transcriptional regulator. The presence of *aadD2* adjacent to genes known to be part of the chromosome confirms its chromosomal location. In addition, repeated attempts to transfer resistance to aminoglycosides and to beta-lactams from *B. clausii* strains to *E. faecalis* JH2-2, *E. faecium* HM1070, and *B. subtilis* UCN19 by conjugation were unsuccessful.

Analysis of the putative promoter of *aadD2* from *B. clausii* SIN. There was a discrepancy between the presence of *aadD2* in all strains of *B. clausii* studied and the various levels of aminoglycoside resistance of the strains. We thus amplified and analyzed a DNA sequence of nearly 200 bp upstream from the *aadD2* genes of *B. clausii* SIN (resistant to aminoglycosides), NR, OC, T, and DSM8716 (susceptible to aminoglycosides). Potential −35 and −10 consensus sequences were identified, one of which may be the recognition site for a DNA-dependent RNA polymerase (Fig. 3). In all strains, one promoter sequence was confidently predicted (prediction score, 0.93). Comparison of the sequences revealed three nucleotide substitutions in the *B. clausii* SIN sequence. Two of these substitutions were located in putative −35 and −10 regions. A C-to-A mutation rendered the −35 sequence (TTAGCA) identical to the consensus promoter sequences of *E. coli* and *B. subtilis* (9, 19), which might result in a stronger promoter. Another mutation was located in the −10 sequence (Fig. 3). This mutation led to the prediction (score, 0.95) of an additional putative promoter. The consequence, if any, of the third substitution remains unknown.

Although the origin of *B. clausii* SIN is not known, the identity of the *Sma*I restricted DNA profiles of strains composing Enterogermina strongly supports the notion that SIN is a derivative of one of the other *B. clausii* probiotic strains or of a common parental strain.

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