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Gene Cluster in *Micromonospora echinospora* ATCC15835 for the Biosynthesis of the Gentamicin C Complex

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Gentamicin is a 4,6-disubstituted aminocyclitol antibiotic complex synthesised by some members of the actinomycete genus *Micromonospora*. In a search for the gentamicin biosynthetic gene cluster we identified, using a cosmid library approach, a region of the *M. echinospora* ATCC15835 chromosome that encodes homologues of aminoglycoside biosynthesis genes including *gntB*–a close homologue of the 2-deoxy-*scyllo*-inosose synthase gene (*btrC*) from butirosin-producing *Bacillus circulans*. Insertional inactivation was achieved by homologous recombination with an internal *gntB* fragment-containing suicide plasmid, delivered by conjugal transfer from *Escherichia coli*. *gntB* disruptants were gentamicin non-producing mutants as assayed by an ELISA antibiotic detection system, proving the association of *gntB* (or a downstream region) with gentamicin biosynthesis. The function of some open reading frames within the cluster, predicted by nucleotide database homology searching, is discussed with regards to their potential roles in gentamicin biosynthesis. The discovery of this genetic region represents the first report of a gene cluster involved in the biosynthesis of a 4,6-disubstituted aminocyclitol antibiotic.

The gentamicin antibiotic was discovered in 1963 and for forty years has been a useful agent for the treatment of severe bacterial infection[1]. In addition to its use as an antibacterial agent, the potential anti-viral properties of some gentamicin-conjugants have recently been demonstrated[2], as has the ability of highly related structures to specifically bind catalytic RNAs[3,4] and small ribozymes *in vitro*, suggesting possible future roles in the development of novel gentamicin-like drugs that specifically target RNA[5].

Gentamicin is a water soluble aminocyclitol-containing aminoglycoside antibiotic complex, consisting predominantly of three major components: gentamicin C1, C1a and C2. The C1, C1a and C2 components (gentamicin Cs) are composed of a central di-aminogenous cyclitol (2-deoxystreptamine (2DOS)) 4,6-disubstituted with the auxiliary sugars garosamine and purpurosamine. Numerous biochemical studies have attempted to elucidate the biosynthetic route to the gentamicin Cs, and a branched pathway has been proposed based on precursor feeding studies of blocked mutants[6~8]. A complete mechanistic pathway is yet to be described, however, and further work is required to clarify the proposed biosynthetic route to the gentamicin Cs.

The gentamicin Cs act by binding to the decoding region of the aminoacyl-tRNA site of the ribosome, inhibiting translocation of the nascent peptide and decreasing translational accuracy[5]. Bacterial self-resistance to the gentamicin components is achieved by specific methylation of ribosomes, presumably catalysed by the GrmA gene product[9]. Prior to this report the *grmA* gene (and a short tandem region of unknown function) were the only reported genes believed to be associated with the gentamicin biosynthesis gene cluster.

Genetic studies have facilitated the study of diverse antibiotic biosynthesis pathways. Of particular relevance, the gene cluster of the 4,5-disubstituted aminocylcitol antibioitic butirosin has recently been identified and

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Table 1. Bacterial strains and plasmids used in this study.

Bacterial strain	**Source**	**Phentotypic/Genotypic Notes**
E. coli Top 10	Invitrogen	F^-, *mcrA*, Δ(*mrr-hsdRMS-mcrBC*), f80*lacZ*DM15, Δ*lacX74*, *deoR*, *recA1*, *araD*139, Δ (*ara-leu*)7697, *galU*, *galK*, *rpsL* (Str^R), *endA1*, *nupG*
E. coli ET12567/pUB307	(Flett *et al.*, 1997)	*Dam-13*::Tn9, *dcm-6*, *hsdM, hsdS,* pUB307
M. echinospora ATCC15835	(Weinstein *et al.*, 1964)	Wild type *Micromonospora* species used industrially for genatmicin C complex production.
M. echinospora ATCC31119 ("Daum's Idiotroph")	(Daum *et al.*, 1977)	Mutant of *M. echinospora* ATCC15835 blocked in early stage of gentamicin production. Produces gentamicin if 2DOS is added to the fermentation medium pre-idiophase.
M. echinospora *Me*ORF4::pSPRU27a	This study	*M. echinospora* ATCC15835 derivative containing an insertionally inactivated *gntB* gene, disrupted by plasmid pSPRU27. Gentamicin non-producing mutant.
Plasmid name	**Source and reference**	**Properties and uses**
pSPWAR001	This study	SuperCos cosmid vector containing a ca. 39.5 kb insert of *M. echinospora* ATCC15835 chromosomal DNA.
pSPRA2350	(Hosted *et al.*, 2001)	*aac(3)IV*, ColE1 ori, *oriT* RK2. *E. coli*-replicating, actinomycete non-replicating ("suicide") vector. Used for generating single crossover insertion mutations in actinomycetes

allowed more detailed studies into the biosynthesis of this aminoglycoside[10]. The work reported here is the first description of the gentamicin gene cluster in *M. echinospora* ATCC15835, and indeed of the first gene cluster known to be involved in the biosynthesis of a 4,6-disubstituted aminocyclitol aminoglycoside. An *E. coli* cosmid library of *M. echinospora* ATCC15835 DNA was screened by the PCR, using primers for the *grmA* gene as a target for the biosynthetic gene cluster (antibiotic self-resistance and biosynthesis genes are often clustered chromosomally[11]. Sequence analysis of the single PCR-positive cosmid led to the identification of a number of ORFs with homology to known aminoglycoside biosynthesis, resistance and regulatory genes. Gene disruption by homologus recombination of a predicted biosynthetic gene yielded a gentamicin non-producing mutant, proving the association of the gene cluster with the biosynthesis of the gentamicin Cs.

Materials and Methods

Bacterial Strains, Culture Conditions and Plasmids

Bacterial strains used in this study are listed in Table 1. *E. coli* strains were cultured in LB medium at 37°C, in liquid or on agar. *Micromonospora* strains were cultured on ATCC172 agar at 30°C. Selected details of the plasmids and cosmid used in this study are listed in Table 1.

DNA Manipulations

Plasmid DNA was extracted from *E. coli* using the QIAprep Spin Plasmid Kit (Qiagen). Cosmid isolations were achieved using the Qiagen Maxi Kit. For the isolation of high molecular weight chromosomal DNA, micro-monosporas were cultivated, lysed and processed essentially as detailed in DAIRI *et al.*[12]. PCR amplifications were carried out essentially as directed by SAMBROOK *et al.*[13]. All PCR amplifications were carried out under identical

cycling conditions: 95°C for 5 minutes followed by 30 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute. A final extension step of 72°C for 10 minutes was used.

Cosmid Library Preparation

The *E. coli* cosmid library of *M. echinospora* ATCC15835 chromosomal DNA was constructed using cosmid vector SuperCos1 (Stratagene) and the instructions of the manufacturer. Packaging was performed with Gigapack II XL packaging extract (Stratagene) as specified by the manufacturers. The library was amplified in Stratagene SURE cells; 1000 clones were picked and individually screened for the *grmA* gene using the primer pair grm1A (5′-GCTCTGCGCCGCGCGATG-3′) and grm2A (5′-CGAGAGACTTGGTCG-3′). Agarose gel electrophoresis of PCR products identified a single positive clone, pSPWAR001.

Cosmid Sequencing

DNA sequencing of a cosmid pSPWAR001 sub-library was conducted by Lark Technologies Inc, Houston, TX, using Big Dye Terminator Cycle sequencing Kits (Applied Biosystems) and were analysed using 96 lane ABI377 machines. Each of 768 colonies was sequenced with pUC forward and reverse oligonucleotide primers, generating 1536 reads in total. Sequencing reads were visualised using Chromas, and aligned using the EditSeq and SeqMan programs of DNASTAR. One hundred percent of the generated consensus sequence was double stranded. Sequences were submitted to Genbank under the following accession number: AY524043.

In Silico Analysis

ORF analysis was conducted using the Artemis program. The predicted amino acid sequences of all ORFs were analysed in BLASTP. Regions of secondary structure (dyad symmetries) within the cosmid clone were predicted using the Clone program where confidence was given to hairpin loops with energy of $-25\,\mathrm{kcal\,mol^{-1}}$ or less.

Creation of Disruption Cassette

Plasmid pSPRU27 was created as follows: a 525 bp internal fragment of *gntB* was amplified from pSPWAR001 DNA using primers *gntB*KOFPacI (5′-GGCCTTAATTAATGTTCGGCGCGGAACTGGTC-3′) and *gntB*KORXbaI (5′-CTCTTCTAGAGCATGTAGCAGAGCAGCAGCAGCAT-3′). *Pac* I and *Xba* I recognition sites were incorporated into the PCR primers (underlined). The PCR-amplified fragment was digested simultaneously with enzymes *Pac* I and *Xba* I (New England Biolabs) and ligated with the similarly treated actinomycete suicide vector pSPRA2350[14] to form vector pSPRU27. Plasmid pSPRU27 DNA was used to electroporate *E. coli* ET12567 cells containing plasmid pUB307[15]. Transformants were selected on LB agar containing apramycin ($50\,\mu\mathrm{g\,ml^{-1}}$), kanamycin ($50\,\mu\mathrm{g\,ml^{-1}}$) and chloramphenicol ($25\,\mu\mathrm{g\,ml^{-1}}$). *E. coli* transformant ETpSPRU27 was generated in this way, and used for subsequent conjugations with *M. echinospora* ATCC15835.

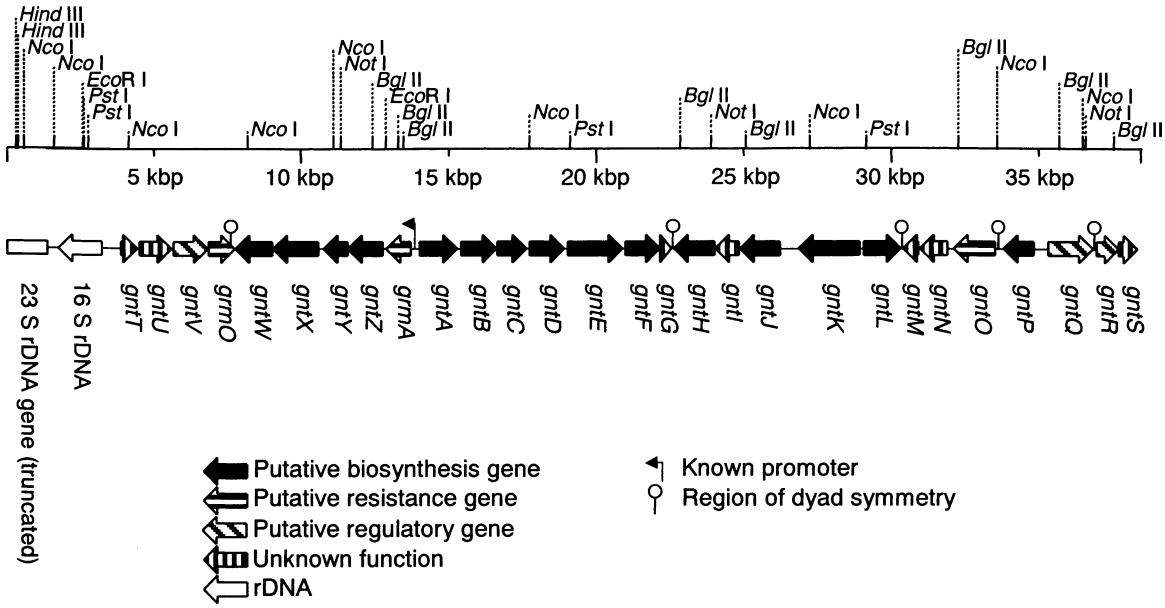
Conjugal Transfer of pSPRU27 to *M. echinospora* ATCC15835

E. coli donor strain ETpSPRU27 was prepared essentially as described in BIERMAN *et al.*[16]. *M. echinospora* ATCC15835 recipient cells were grown to stationary phase (*ca.* 10 days) in ATCC172 medium. Cells ($200\,\mu$l) were transferred to fresh 10 ml aliquots of ATCC172 medium and incubated with shaking at 200 rpm at 30°C for 96 hours. Cells (10 ml) were homogenised and pelleted by centrifugation with three subsequent washes in TSB broth. Cells were finally resuspended in 2 ml TSB and stored on ice until needed. The similarly washed *E. coli* strain ETpSPRU27 ($150\,\mu$l) was added to $450\,\mu$l *M. echinospora* cells and mixed for three seconds at low speed on a vortex. Aliqouts of the mating mixture ($150\,\mu$l) were plated onto 25 ml modified AS-1 medium[17] in 9 cm petri dishes and incubated at 30°C for 16 hours. Plates were then overlaid with 1 ml sdw containing 0.5 mg nalidixic acid and 1.25 mg apramycin. Incubation was continued at 30°C and apramycin/nalidixic acid-resistant micromonosporas were visible after 96 hours. Apramycin/nalidixic acid-resistant colonies were picked to modified AS-1 plates containing $50\,\mu\mathrm{g\,ml^{-1}}$ apramycin and $20\,\mu\mathrm{g\,ml^{-1}}$ nalidixic acid to remove any *E. coli* resistant background contaminants before fermentation.

Clarification of Disruption of *gntB* by pSPRU27

Chromosomal DNA was isolated from *M. echinospora* ATCC15835 exconjugant M*egntB*::pSPRU27a. Probe ORF4Pr was synthesised by the PCR using primers *gntB*Prf (5′-TGTTCGGCGCGGAACTGGTC-3′) and *gntB*4Prr (5′-CGTACATCGTCTCGTCGTCGTA-3′) and purified pSPWAR001 cosmid as template DNA, incorporating DIG dUTP as directed by the manufacturer (Roche). Southern hybridisation with probe *gntB*Pr was performed at high stringency on agarose gel-separated chromosomal DNA that had been transferred to nitrocellulose filters after digestion with enzymes *Bgl* II and *Nco* I separately. The estimated sizes of the hybridising bands were compared to

Fig. 1. Orientation of ORFs in the gentamicin biosynthesis gene cluster.



those predicted before and after integration of pSPRU27 internal to *gntB*.

Fermentation of *Micromonospora* Strains for Gentamicin C Production

Fermentation of strain M*egntB*::pSPRU27a was carried out using *Micromonospora* seed[18] and fermentation media[19] and the methods described in WAGMAN *et al.*[19]. Strain *M. echinospora* ATCC31119 (Daum's idiotrophic gentamicin non-producing mutant) was fermented in tandem as a negative control.

Isolation and Assay of Gentamicin Complex Components

The gentamicin complex components were obtained from whole fermentation broths as detailed in DAUM *et al.*[18]. Levels of the gentamicin C components were assayed using the Innofluor® GM assay system (Seradyn, Inc.) and a TDx™ analyser (Bio-Stat Diagnostic Systems).

Results and Discussion

Cosmid pSPWAR001 contains a 38,146 bp region of the *M. echinospora* ATCC15835 chromosome; analysis of predicted protein coding regions revealed the inserted DNA contained 28 ORFs, all of which appear complete (Figure 1). The similarity of predicted primary amino acid sequences of each putative ORF to known sequences, and selected other details, are described in Table 2. Orientation of the ORFs, along with the presence of potential transcriptional terminators, suggest that unlike the butirosin cluster, the gentamicin biosynthesis gene cluster is likely not transcribed as a single monocistronic transcript.

The cluster contained a complete 16S rRNA gene, and was also flanked by the 1366 bp 3′ region of a 23S rRNA gene. The self-resistance gene for the gentamicin complex is positioned in close proximity to the site of action of its gene product, this proximity has not been reported previously in other aminoglycoside producers.

In order to investigate whether genes in the cluster were involved in the biosynthesis of the gentamicin Cs, *gntB* (a close homologue of the 2-deoxy-*scyllo*-inosose synthase gene *btrC* from *B. circulans*) was insertionally inactivated with plasmid pSPRU27. The *aac(3)IV* gene was used as a selectable marker for homologous recombinants, and so traditional bioassay methods for the detection of gentamicin C production were not of use here (the *aac(3)IV* gene product inactivates the gentamicin Cs by acetylation). In preference we adopted the Innofluor® ELISA system as it detects the gentamicin C components, and the respective biologically inactive *N*-acetyl derivatives of each generated by the action of the *aac(3)IV* gene product (manufacturer's personal communication). In results to be fully reported elsewhere, the Innofluor® system detected the *N*-acetyl

Table 2. Properties of ORFs identified in the pSPWAR001 cosmid insert.

Gene / %G+C	ORF length (AA)	Position in cluster	Protein structural motifs and (Pfam accession number)	Most similar database sequence (%AA identity / %AA similarity and length of AA sequence overlap)	Predicted role
23 S rDNA	-	1349 → 1	None	-	23 S rRNA sub-unit (3' truncated)
16 S rDNA	-	3208 → 1692	None	-	16 S rRNA sub-unit
gntT / 72.6	180	3837 → 4376	None	No typical homology	Unknown
gntU / 72.0	360	4467 → 5546	tRNA synthetase 1b (00579)	*Treponema pallidum* TrsA (61/80 329)	Biosynthesis of tryptophanyl tRNA
gntV / 71.3	386	5613 → 6770	Queuine tRNA-ribosyltransferase (01702)	*Zymomonas mobilis* Tgt (35/62 362)	Regulation
grmO / 74.5	298	6802 → 7695	None	*M. rosea* GrmA (29/55 270)	Resistance
gntW / 72.9	418	8962 → 7709	Aminotransferase class-III (00202)	*B. circulans* BtrB (32/58 416)	Aminotransferase
gntX / 71.7	508	10517 → 8994	GMC oxidoreductase (00732)	*Sinorhizobium meliloti* putative dehydrogenase (30/54 483)	Oxidoreductase / dehydrogenase
gntY / 76.5	270	11499 → 10690	Short chain dehydrogenase (00106)	*Leishmania major* hypothetical protein (34/56 231)	Dehydrogenase
gntZ / 72.3	392	12689 → 11514	Glycosyltransferase 1 (00534)	*B. circulans* BtrM (26/48 372)	Glycosyltransferase
grmA / 65.0	275	13622 → 12798	None	GrmA (100/100 275)	Resistance
gntA / 69.7	421	13917 → 15179	DegT/DnrJ/EryC1/StrS family (01401) Cys/Met metabolism PLP-dependent enzyme (01053)	*S. griseus* StsC (55/75 421)	L-glutamine: 2-deoxy-*scyllo* inosose aminotransferase
gntB / 68.8	398	15314 → 16507	3-dehydroquinate synthase (01761)	*B. circulans* BtrC (38/61 367)	2-deoxy-*scyllo*-inosose synthase
gntC / 71.5	328	16542 → 17525	Oxidoreductase family, NAD-binding Rossmann fold (01408) Oxidoreductase family, C-terminal alpha/beta domain(02894)	*Bacillus halodurans* putative NADH-dependent dehydrogenase (26/50 320)	Dehydrogenase
gntD / 68.5	391	17614 → 18786	Glycosyltransferase 1 (00534)	*B. circulans* BtrM (27/50 385)	Glycosyltransferase
gntE / 65.5	635	18863 → 20767	None	*Lactococcus lactis* putative Fe-S oxidoreductase (20/47 609)	Oxidoreductase / methyltransferase
gntF / 68.6	391	20851 → 22023	DegT/DnrJ/EryC1/StrS family (01401) Cys/Met metabolism PLP-dependent enzyme (01053)	*S. griseus* StsC (38/59 386)	Aminotransferase

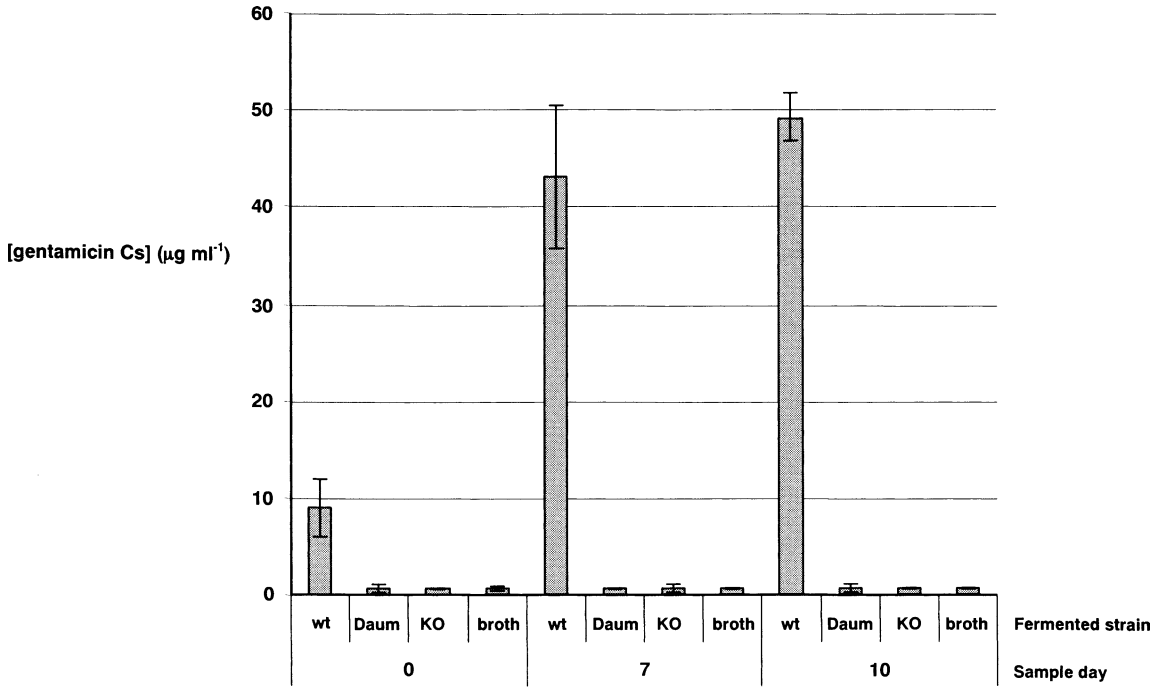
Table 2. Continued.

Gene / %G+C	ORF length (AA)	Position in cluster	Protein structural motifs and (Pfam accession number)	Most similar database sequence (%AA identity / %AA similarity and length of AA sequence overlap)	Predicted role
gntG / 65.9	124	22031 → 22402	None	*H. pylori* conserved hypothetical protein (49/66 123)	Unknown
gntH / 66.1	446	23832 → 22495	Aminotransferase class-III (00202)	*Xanthamonas campestris* putative aminotransferase (29/58 396)	Aminotransferase
gntI / 69.6	269	24694 → 23888	Aminoglycoside phosphotransferases family (01636)	*M. chalcea* aminoglycoside-O-phosphoryl-transferase type Vc (44/63 261)	Resistance / Biosynthesis
gntJ / 65.0	451	26084 → 24732	Aminotransferase class-III (00202)	*B. circulans* BtrB (24/50 430)	Aminotransferase
gntK / 68.7	639	28636 → 26720	MoaA_NifB_PqqE	Fortimicin KL 1 methyltransferase (57/76 553)	Methyltransferase
gntL / 70.3	415	28886 → 30130	Aminotransferase class-III (00202)	*B. circulans* BtrB (30/55 430)	Aminotransferase
gntM / 69.4	171	30733 → 30221	None	*Kitasatospora griseorubida* hypothetical protein (41/65 166)	Unknown
gntN / 73.6	312	31723 → 30788	WD-repeat containing protein	*Amycolatopsis orientalis* putative WD-repeat containing protein (50/72 311)	Unknown
gntO / 71.1	469	33324 → 31918	Sugar transport domain (00083)	*S. coelicolor* putative membrane efflux protein (45/75 465)	Resistance
gntP / 71.0	341	34639 → 33617	Zinc-binding dehydrogenase (00107)	*Pseudomonas aeruginosa* 2,3-butanediol dehydrogenase (31/55 338)	Dehydrogenase
gntQ / 70.2	505	35120 → 36634	Na^+/H^+ exchanger/antiporter (00999)	*Streptomyces toyocaensis* StaN putative membrane integral ion antiporter (28/53 409)	Regulation
gntR / 72.0	246	36756 → 37493	Thiamine biosynthesis (02568) Asn synthase (00733)	*Ralstonia solanacearum* hypothetical signal peptide protein (65/83 223)	Regulation
gntS / 72.0	213	37499 → 38137	None	*Caulobacter crescentus* conserved hypothetical protein (67/83 209)	Unknown

gentamicin C components in fermentation mixtures that otherwise gave negative results in standard diffusion plate bioassays against gentamicin-sensitive *E. coli* strains. Levels of the gentamicin Cs in the inactivated mutant were close to those in the non-producing idiotrophic mutant and to the broth only control, proving the association of *gntB*, or one of the genes disrupted by the polar effect of *gntB* disruption, with biosynthesis of the gentamicin Cs.

Predicted roles of a number of genes within the gentamicin biosynthetic gene cluster have been proposed based on homology searching, and these are shown in Figure 3. Interestingly, apparently co-ordinately transcribed operon *gntABCDEFG* contained a number of genes with predicted involvement in 2DOS biosynthesis. This putative operon contains two *stsC* homologues; the possibility of two separate enzymes acting during the biosynthesis of 2DOS has been raised previously[20]. Preceding the second transamination to form 2DOS, the singly aminated cyclitol is believed to undergo dehydrogenation at the C3 position of the cyclitol ring to yield a suitable keto intermediate for

Fig. 2. Gentamicin C production by wild type and mutant strains of *M. echinospora*.



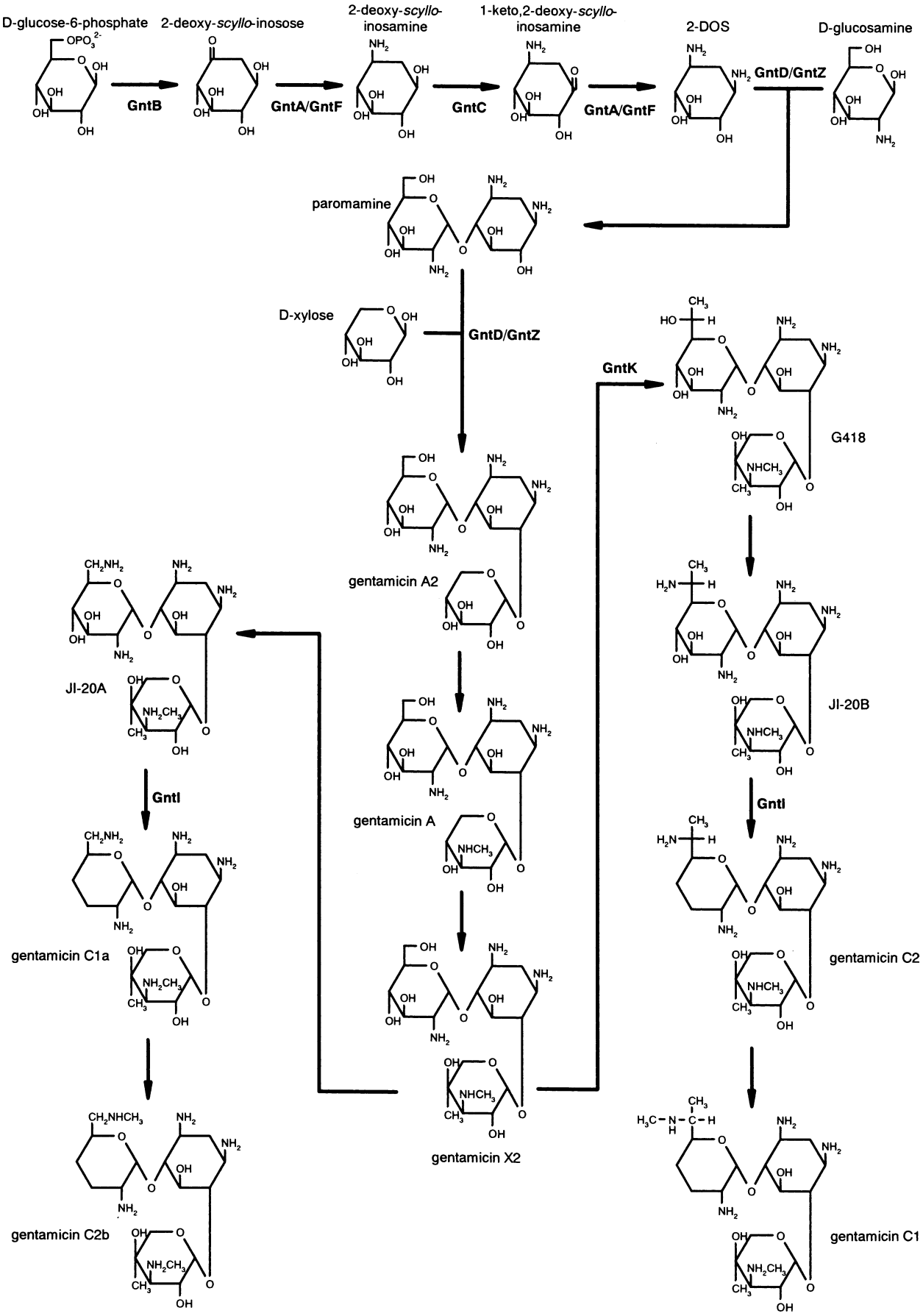
wt, wild type *M. echinospora* ATCC15835; Daum, Daum's idiotrophic mutant; KO, *M. echinospora* M*egntB*::pSPRU27a; broth, broth only control.

aminotransfer. The GntC protein shares significant similarity with a predicted NADH-dependent dehydrogenase from *Bacillus halodurans*[21], and significant similarity is also shared with a *myo*-inositol dehydrogenase from *Mesorhizobium loti*[22]. Pfam confidently predicts the presence of three structural motifs: an *N*-terminus NAD-binding Rossman fold, and a *C*-terminal oxidoreductase alpha-beta domain, which further support the role of the putative GntC protein as a dehydrogenase. These data taken together suggest GntC may act 2DOS biosynthesis by reducing the *myo*-inositol like molecule 2-deoxy:*scyllo*-inosamine to generate a keto intermediate for the second aminotransfer reaction.

Two glycosyltransfer events have been predicted for gentamicin biosynthesis, namely the addition of D-glucosamine to 2DOS to form the pseudodisaccharide intermediate paromamine, and the subsequent addition of D-xylose to paromamine to form gentamicin A2. Two glycosyltransferases have been identified within the gentamicin cluster, GntD and GntZ, both of which share significant similarity with the *B. circulans* BtrM protein[10]. Niether GntD, GntZ nor BtrM contain the glycosyltransferase protein motifs proposed by WATANABE *et al.*[23] or CUNDLIFFE *et al.*[24]. The mechanism of glycosyltransfer employed in the synthesis of 2DOS-containing ACAGs differs from a number of other secondary metabolic glycosyltransferase reactions, in that the reaction apparently does not require the activation of either participating molecule by nucleotide (NDP) activation[25]. GntD, GntY and BtrM appear to form a group of glycosyltransferase enzymes unique from other secondary metabolic glycosyltransferases.

The GntK protein shares 57% identity with the Fms7 gene product of *M. olivasterospora*. Fms7 catalyses the 6′-*C*-methylation of the purpurosamine moiety of fortimicin KL_1, converting it to fortimicin KK_1, as deduced from genetic complementation studies of *M. olivasterospora* mutants blocked in fortimicin KK_1 biosynthesis[12,26]. The step catalysed by Fms7 is specific to organisms of the genus *Micromonospora* as the glycosidic component of the pseudodisaccharide present in streptomycete-produced contains an *N*-methyl group rather than a *C*-methyl at the

Fig. 3. Bosynthetic route to the gentamicin C complex. Predicted roles of gentamicin biosynthesis genes are shown.



6′-C[12]. Two *C*-methylation steps occur during the biosynthesis of the gentamicin Cs, namely the conversions of gentamicin A to gentamicin X_2 and of gentamicin X_2 to antibiotic G418. The latter of these steps represents a 6′-*C*-methylation analogous to that of the Fms7 reaction, and hence GntK may participate in this step.

In addition to *grmA*, the pSPWAR001 insert appears to encode at least two additional resistance genes. The existence of a second resistance methyltransferase gene (*grmO*) is supported by previous workers[27] but GntN is the first presumed aminoglycoside export protein known to be physically linked with a 2DOS containing aminoglycoside biosynthetic cluster. The *gntI* gene may play a resistance, regulatory or biosynthesis role. The predicted GntI protein shares significant similarity with a number of aminoglycoside-*O*-phosphoryltransferases of the APH(3′)-Vc family–enzymes that inactivate certain aminoglycosides *via* transfer of the γ-phosphate group from ATP to the 3′ hydroxyl moiety of the acceptor aminoglycoside. It is unlikely that the *gntI* gene acts as major resistance determinant to the gentamicin Cs as each lack the 3′ hydroxyl group which acts as an acceptor for phosphoryltransfer from ATP. GntI may have a regulatory role in gentamicin biosynthesis as there is a known phylogenetic link between APH enzymes and protein kinases[28], and APH(3′)-III enzymes (that are closely related to the APH(3′)-V family) can phosphorylate some protein and peptide substrates. GntI contains the common site motif (Lys_{44}, Glu_{60}, Asp_{195}, Asp_{208}) that resembles the active site residues found in the Ser/Thr/Tyr protein kinase family[29]. Tyrosine kinases have previously been implicated in the regulation of streptomycin biosynthesis in *S. griseus*[30]. Involvement in a biosynthetic step is also feasible, similar to that proposed for the product of the *fms8* in fortimicin biosynthesis in *M. olivasterospora*. DAIRI *et al.*[26] showed *fms8* was able to complement mutants deficient in the double dehydroxylation step of FTM-A biosynthesis (FTM-KK1 to FTM-AP). The biosynthetic step could, however, also be carried out by the *M. chalcea* and *S. fradiae* APH(3′)-V phosphotransferases. This led to the conclusion that the double dehydroxylation step is preceded by a 3′ phosphorylation step, as is catalysed by typical APH(3′)-V enzymes. Two very similar steps have been proposed in gentamicin biosynthesis and hence the protein could effect the conversion of JI20A to gentamicin C1a, and JI20B to gentamicin C2. The nucleotide sequence of *fms8* has not been submitted to the NCBI database, so comparison of the GntI and Fms8 sequences has not been possible.

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References

1) WEINSTEIN; M. LUEDEMANN, E. M. ODEN, G. H. WAGMAN, J. P. ROSSELET, J. A. MARQUEZ, C. T. CONIGLIO, W. CHARNEY, H. L. HERZOG & J. BLACK: Gentamicin, a new antibiotic complex from *Micromonospora*. J. Med. Chem. 122: 463～464, 1963
2) LITOVCHICK, A.; A. G. EVDOKIMOV & A. LAPIDOT: Arginine-aminoglycoside conjugates that bind to HIV transactivation responsive element RNA *in vitro*. FEBS Lett. 445: 73～79, 1999
3) ROGERS, J. & J. DAVIES: The pseudodisaccharides: a novel class of group I intron splicing inhibitors. Nucleic Acids Res. 22: 4983～4988, 1994
4) EUBANK, T. D.; R. BISWAS, M. JOVANOVIC, A. LITOVCHICK, A. LAPIDOT & V. GOPALAN: Inhibition of bacterial RNase P by aminoglycoside-arginine conjugates. FEBS Lett. 511: 107～112, 2002
5) SCHROEDER, R.; C. WALDSICH & H. WANK: Modulation of RNA function by aminoglycoside antibiotics. Embo. J. 19: 1～9, 2000
6) TESTA, R. T. & B. C. TILLEY: Biotransformation, a new approach to aminoglycoside biosynthesis: II. Gentamicin. J. Antibiotics 29: 140～146, 1976
7) TESTA, R. T. & B. C. TILLEY: Biosynthesis of sisomicin and gentamicin. Jpn. J. Antibiotics 32 (Suppl.): S47～S59, 1979
8) KASE, H.; Y. ODAKURA & K. NAKAYAMA: Sagamicin and the related aminoglycosides: fermentation and biosynthesis. I. Biosynthetic studies with the blocked mutants of *Micromonospora sagamiensis*. J. Antibiotics 35: 1～9, 1982
9) KELEMEN, G. H.; E. CUNDLIFFE & I. FINANCSEK: Cloning and characterization of gentamicin-resistance genes from *Micromonospora purpurea* and *Micromonospora rosea*. Gene 98: 53～60, 1991
10) OTA, Y.; H. TAMEGAI, F. KUDO, H. KURIKI, A. KOIKE-TAKESHITA, T. EGUCHI & K. KAKINUMA: Butirosin-biosynthetic gene cluster from *Bacillus circulans*. J. Antibiotics 53: 1158～1167, 2000
11) MARTIN, M. F. & P. LIRAS: Organization and expression of genes involved in the biosynthesis of antibiotics and other secondary metabolites. Annu. Rev. Microbiol. 43: 173～206, 1989
12) DAIRI, T.; T. OHTA, E. HASHIMOTO & M. HASEGAWA: Self cloning in *Micromonospora olivasterospora* of *fms* genes for fortimicin A (astromicin) biosynthesis. Mol. Gen. Genet. 232: 262～270, 1992
13) SAMBROOK, J.; E. F. FRITSCH & T. MANIATIS: Molecular Cloning, a laboratory manual, Second Edition, Cold Spring Harbor Laboratory Press, NY, 1989
14) HOSTED, T. J.; T. X. WANG, D. C. ALEXANDER & A. C. HORAN: Characterization of the biosynthetic gene cluster for the oligosaccharide antibiotic, Evernimicin, in

Micromonospora carbonacea var. *africana* ATCC39149. J. Ind. Microbiol. Biotechnol. 27: 386～392, 2001

15) Flett, F.; V. Mersinias & C. P. Smith: High efficiency intergeneric conjugal transfer of plasmid DNA from *Escherichia coli* to methyl DNA-restricting streptomycetes. FEMS Microbiol. Lett. 155: 223～229, 1997

16) Bierman, M.; R. Logan, K. O'Brien, E. T. Seno, R. N. Rao & B. E. Schoner: Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. Gene 116: 43～49, 1992

17) Alexander, D. C.; D. J. Devlin, D. D. Hewitt, A. C. Horan & T. J. Hosted: Development of the *Micromonospora carbonacea* var. *africana* ATCC 39149 bacteriophage pMLP1 integrase for site-specific integration in *Micromonospora* spp. Microbiology 149: 2443～2553, 2003

18) Daum, S. J., D. Rosi & W. A. Goss: Mutational biosynthesis by idiotrophs of *Micromonospora purpurea*. II. Conversion of non-amino containing cyclitols to aminoglycoside antibiotics. J Antibiotics 30: 98～105, 1977

19) Wagman, G. H.; R. T. Testa & J. A. Marquez: Antibiotic 6640. II. Fermentation, isolation, and properties. J. Antibiotics 23: 555～558, 1970

20) Ahlert, J.; J. Distler, K. Mansouri & W. Piepersberg: Identification of *stsC*, the gene encoding the L-glutamine:*scyllo*-inosose aminotransferase from streptomycin-producing streptomycetes. Arch Microbiol. 168: 102～113, 1997

21) Takami, H.; K. Nakasone, Y. Takaki, G. Maeno, Y. Sasaki, N. Masui, F. Fuji, C. Hirama, Y. Nakamura, N. Ogasawara, S. Kuhara & K. Horikoshi: Complete genome sequence of the alkaliphilic bacterium *Bacillus halodurans* and genomic sequence comparison with *Bacillus subtilis*. Nucleic Acids Res. 28: 4317～4331, 2000

22) Kaneko, T.; Y. Nakamura, S. Sato, E. Asamizu, T. Kato, S. Sasamoto, A. Watanabe, K. Idesawa, A. Ishikawa, K. Kawashima, T. Kimura, Y. Kishida, C. Kiyokawa, M. Kohara, M. Matsumoto, A. Matsuno, Y. Mochizuki, S. Nakayama, N. Nakazaki, S. Shimpo, M. Sugimoto, C. Takeuchi, M. Yamada & S. Tabata: Complete genome structure of the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti*. DNA Res. 7: 331～338, 2000

23) Watanabe, M.; K. Miyake, K. Yanae, Y. Kataoka, S. Koizumi, T. Endo, A. Ozaki & S. Iijima: Molecular characterization of a novel beta1,3-galactosyltransferase for capsular polysaccharide synthesis by *Streptococcus agalactiae* type Ib. J. Biochem. (Tokyo) 131: 183～191, 2002

24) Cundliffe, E.; S. A. Fish, V. T. W. Wilson, A. Gandecha & S. L. Large: Glycosyl transfer during tylosin production in *Streptomyces fradiae*. Dev. Indust. Microbiol. 34: 109～114, 1997

25) Piepersberg, W.: Molecular biology, biochemistry, and fermentation of aminoglycoside antibiotics. *In* Biotechnology of Antibiotics. 2nd edn., pp. 81～161. W. R. Strohl and H von Dohren eds.

26) Dairi, T.; T. Ohta, E. Hashimoto & M. Hasegawa: Organization and nature of fortimicin A (astromicin) biosynthetic genes studied using a cosmid library of *Micromonospora olivasterospora* DNA. Mol. Gen. Genet. 236: 39～48, 1992

27) Ohta, T. & M. Hasegawa: Analysis of the self-defense gene (*fmrO*) of a fortimicin A (astromicin) producer, *Micromonospora olivasterospora*: comparison with other aminoglycoside-resistance-encoding genes. Gene 127: 63～69, 1993

28) Lim, C. K.; M. C. Smith, J. Petty, S. Baumberg & J. C. Wootton: *Streptomyces griseus* streptomycin phosphotransferase: expression of its gene in *Escherichia coli* and sequence homology with other antibiotic phosphotransferases and with eukaryotic protein kinases. J. Gen. Microbiol. 135: 3289～3302, 1989

29) Wright, G. D. & P. R. Thompson: Aminoglycoside phosphotransferases: proteins, structure, and mechanism. Front Biosci. 4: D9～D21, 1999

30) Holt, T. G.; C. Chang, C. Laurentwonter, T. Murakami, J. Garrels, J. E. Davies & C. J. Thompson: Global change in gene expression related to antibiotic synthesis in *Streptomyces hygroscopicus*. Mol. Microbiol. 6: 969～980, 1992