DOI: 10.1002/cbic.201100347

Identification of a Gene Cluster that Initiates Azasugar Biosynthesis in *Bacillus amyloliquefaciens*

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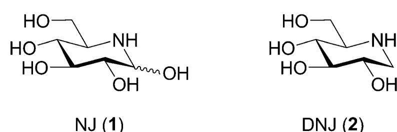
Azasugars feature a ring nitrogen atom instead of an oxygen atom, and are also known as iminosugars, iminocyclitols, or more formally as polyhydroxy derivatives of piperidines or pyrollidines.[1] The first azasugar to be characterized was nojirimycin (NJ, **1**) isolated in 1966 from *Streptomyces* cultures.[2,3] Its derivative, 1-deoxynojirimycin (DNJ, **2**) was initially obtained



through chemical synthesis and later isolated from diverse natural sources.[4,5] These include some *Bacilli* species, in addition to various plants, such as *Albus* (mulberry).[6] Azasugars and their derivatives are potent inhibitors of glycosidases, N-glycohydrolases, phosphorylases, and glycosyltransferases.[7] This activity is typically attributed to the basic ring nitrogen atom, which when protonated may serve as a charge-mimic of glycosyl oxocarbenium ions, or related transition states. Azasugars and their analogues are of interest for a number of applications,[8] including the treatment of type 2 diabetes (miglitol) and lysosomal storage diseases (miglustat).[9] Though the naturally occurring parent microbial azasugars are available through fermentation, synthetic routes to their derivatives typically have the overheads of protection and deprotection. Various synthetic strategies have been and continue to be reported for this class of natural product[5,10] Despite this work, the biosynthetic pathway of azasugars remained unknown with the exception of some insightful results obtained from feeding experiments with labeled precursors, performed nearly 20 years ago.

In the early 1990s, Hardick and co-workers fed stable-isotope-labeled glucose to *Bacillus atrophaeus* and *Streptomyces subrutilus* to establish glucose as a precursor of DNJ.[11] This is in contrast to the biosynthesis of castanospermine and swainsonine, inhibitory polyhydroxy alkaloids with an amino acid biosynthetic origin.[12] Furthermore, it was shown that the carbon skeleton of glucose undergoes inversion, as evidenced by [1-^{13}C]glucose having produced DNJ labeled at C6 (Scheme 1). This implied that DNJ was formed via a C2-*N*-C6 cyclization reaction, operating in the following way. Glucose could readily be drawn out of glycolysis as a fructosyl species, which upon transamination would yield a 2-aminomannitol (**3**). Oxidation of the C6 hydroxyl group would yield a 6-oxo species that would reasonably be expected to rapidly cyclize to mannojirimycin (**4**). Epimerization at the new C-2 (former C-5 of glucose) would produce nojirimycin, yielding 1-deoxynojirimycin after loss of 1-OH and reduction. This proposal, consistent with the results of the labeling studies is presented in Scheme 1. We sought evidence for this proposed pathway, both in terms of the chemical intermediates and the identity of the genes coding for the biosynthetic enzymes. Only recently has this become practical, with the reports of sequenced genomes for the azasugar producing *Bacillus amyloliquefaciens* and *B. atrophaeus*.[13,14]

In this communication we identify three enzymes implicated in the first steps of biosynthesis of DNJ in *Bacillus amyloliquefaciens*. We searched for candidate genes by focusing on those coding for enzymes that could catalyze all or some of the reactions required to convert a fructosyl species to DNJ. While a priori there was no reason to require it, we sought genes which were clustered, and also passed the following criteria. We centered on aminotransferases and redox enzymes and secondarily, gave higher priority when one or more of the gene products exhibited specificity for carbohydrates or carbohydrate-like molecules. A cluster of three genes was identified, designated[13] *gabT1*, *yktc1*, and *gutB1*, coding for putative aminotransferase, phosphatase, and zinc-dependent dehydrogenase enzymes. The *gabT1* gene is a member of the acetyl ornithine aminotransferase family[15] and a translated nucleotide BLAST of *gabT1* against the nr protein database revealed it shares 50 % identity with ValM, the putative aminotransferase involved in validamycin biosynthesis in *Streptomyces hygroscopicus*.[16] The *yktc1* gene is a member of the FIG superfamily,[17] which are metal-dependant phosphatases whose substrates include fructose and inositol phosphates. The final gene in the cluster, *gutB1*, is a member of the medium-chain reductase/dehydrogenase family,[18] which includes iditol and sorbitol dehydrogenases. Taking the putative functions of these genes into consideration along with the results from the labeling studies, we hypothesize that the three enzymes are involved in DNJ biosynthesis as shown in Scheme 1. The GabT1 enzyme could add the amino group to C2 of fructose-6-phosphate, after which Yktc1 removes the phosphate group, yielding 2-aminomannitol (**3**). GutB1 would then oxidize the unmasked hydroxyl group on C6 leading to formation of mannojirimycin (**4**). It is noteworthy to point out that the same gene cluster is also present in *B. atrophaeus* and *B. pseudomycoides*,[14,19] the former species is a known DNJ producer, and we consider the latter to be a candidate azasugar producer on the basis of the presence of the cluster.

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201100347.

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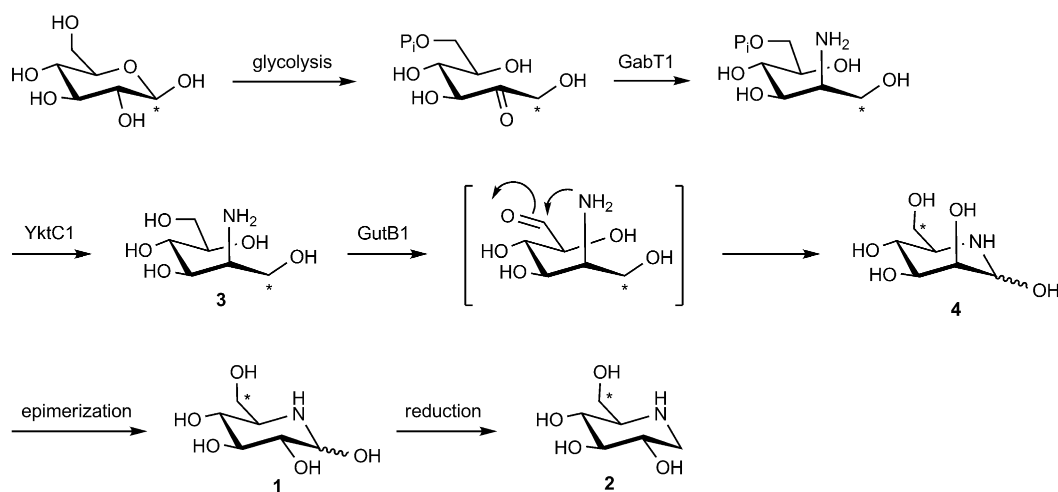

Scheme 1. A summary of the proposed sequence for biosynthesis of **2** based on stable-label isotope feeding studies and the work described in the present study. The asterisk represents the fate of C-1 of glucose based on labeling studies.[11] The enzymes identified in the scheme were initially based on bioinformatic analyses of *B. amyloliquefaciens* and *B. atrophaeus*, as described in the text.

In order to test the proposed function of the *gabT1-yktC1-gutB1* cluster, we initially set out to knock out the *gabT1* gene in *Bacillus amyloliquefaciens*, which according to our hypothesis, would eliminate DNJ production. A plasmid construct was created utilizing the pETBlue-2 vector containing the chloramphenicol acetyltransferase (*cat*) gene and promoter flanked by ~600 bp of the *gabT1* gene corresponding to the 5′- and 3′-ends of the gene. These flanking sequences were used to direct homologous recombination into the *gabT1* locus, and result in disruption with the selectable *cat* marker gene. Transformation of this construct into *B. amyloliquefaciens* resulted in transformants resistant to chloramphenicol.[17] Disruption of the *gabT1* gene within these transformants was further confirmed by colony PCR, using primers complementary to the 3′-end of the *cat* gene and to the 5′-end of the *yktc1* gene. The phenotype of the *gabT1* knockout with respect to DNJ production and the role of the GabT1 enzyme were determined by kinetic and spectroscopic experiments. Cultures of *gabT1*::*cat* and wild-type *B. amyloliquefaciens* were grown in parallel and culture extracts applied to cation-exchange resin (Amberlite IR-120, H^+ form).[11a] Because wild-type *B. amyloliquefaciens* produces DNJ, α-glucosidase will be inhibited by the wild-type culture extract. In contrast, relief of inhibition is anticipated from the knockout *gabT1*::*cat B. amyloliquefaciens* culture extracts. Figure 1 shows the results from the kinetic assay, evidencing relief of glucosidase inhibition by the knockout. Interestingly, we find that a control assay based on parallel fractionation of culture media from the DNJ *nonproducer B. subtilis 168* shows a level of activity comparable to the *gabT1* knockout. We cautiously suggest that the low level of DNJ produced in *B. amyloliquefaciens* results in extracts that are dominated by other compounds, some of which may be inhibitory at high concentrations. What is crucial to note however, is that knockout of *gabT1* results in markedly less inhibition of maltase, and this in turn suggests a crucial role of the *gabT1* gene in DNJ biosynthesis as the putative transaminase. Support for this hypothesis was further obtained by chemical complementation, which involved growing the knockout strain in the presence of the proposed precursor 2-aminomannitol (**3**; Scheme 1). If this compound is indeed an intermediate in the biosynthetic pathway, DNJ production should be reinstated in the knockout culture grown in its presence. This proved to be the case; inhibition of α-glucosidase was restored to nearly the same level as seen for the wild-type (Supporting Information), supporting 2-aminomannitol as a putative DNJ precursor molecule. Culture extracts of the wild-type and *gabT1* knockout were then analyzed with HPLC/(+)ESI-MS/MS (Figure 2). The m/z 164 $[M+H]^+$

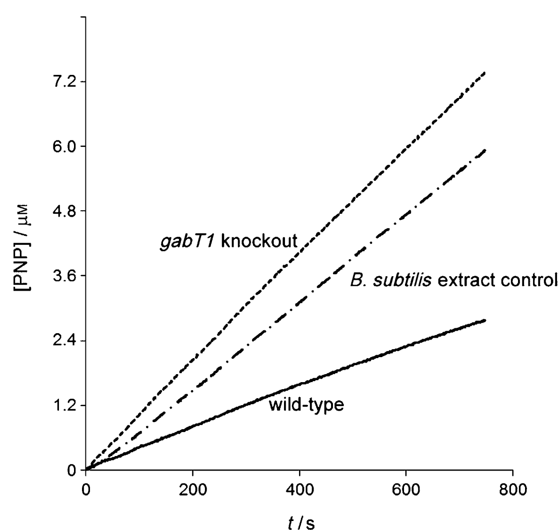

Figure 1. Knockout of DNJ production in *B. amyloliquefaciens*. The data show the velocity of maltase-catalyzed hydrolysis of α-PNP glucoside in the presence of *B. amyloliquefaciens* (or *B. subtilis*) culture extracts. The velocity of the maltase reaction was considerably slower in the presence of extracts from wild-type *B. amyloliquefaciens* compared to the *gabT1* knockout. The *B. subtilis* control (middle line) shows nearly the same velocity as the knockout, and demonstrates that extracts from a non-DNJ-producing strain may show very modest nonspecific inhibition.

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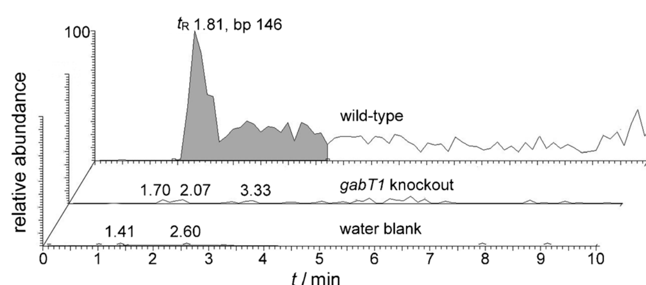

Figure 2. HPLC/(+)ESI-MS/MS m/z 164→m/z 146 [base peak (bp)] product ion mass chromatograms for wild-type (top), *gabT1* knockout (middle) and water blank. Additional MS analytical data and methods are included in the Supporting Information.

ion of DNJ and its MS/MS product ions were present in the wild-type culture extract but were not present in the knockout. Controls with authentic DNJ showed virtually identical MS/MS spectra (Supporting Information). The combined kinetic, chemical complementation, and spectroscopic results provide strong support that GabT1 is a critical enzyme in DNJ biosynthesis, likely removing fructose-6-phosphate from glycolysis via transamination.

Next, we tested the hypothesis that GabT1, Yktc1, and GutB1 are sufficient to produce mannojirimycin. By expression of the three enzymes in *E. coli*, which does not produce azasugars, we should be able to confer synthesis of mannojirimycin in this species if our hypothesis was correct. The *gabT1*, *yktc1* and *gutB1* genes were amplified from the *B. amyloliquefaciens* genome as one PCR product and cloned into the *E. coli* expression vector pETBlue-2. After sequencing, the construct was used to transform RosettaBlue(DE3) cells, which were subsequently grown in low-salt LB medium, and induced at an OD_{600} of 0.75 with IPTG (1 mM). An empty vector control culture was grown in parallel. Culture supernatants were purified on Amberlite IR-120 resin (H^+ form), followed by Dowex 1×8 resin (OH form). Samples were then reduced with $NaBD_4$, in order to convert mannojirimycin (MJ) to 1-[^{2}H]deoxymannojirimycin, (DMJ) a stable species that can be analyzed by mass spectrometry. Both MJ and DMJ were initially identified via kinetic assay in the following way. Fractions were added to assay mixtures consisting of jack bean α-mannosidase and PNP-α-D-mannoside.[20] Significant mannosidase inhibition was detected only from the *E. coli* cultures harboring the *gabT1-yktc1-gutB1* gene cluster and not in the control culture containing empty vector (Figure 3). Aliquots of $NaBD_4$-treated culture extracts were analyzed via HPLC/ESI-MSn (Supporting Information). The expected m/z 165 $[M+H]^+$ ion of 1-[^{2}H]deoxymannojirimycin and its MS/MS product ions were detected from the gene-cluster expression construct transformant but were not detected in the empty-vector transformant. The results indicate that an *E. coli* expression system containing the *gabT1*, *yktc1*, and *gutB1* genes can produce mannojirimycin, corroborating the results of knocking out *gabT1* in *B. amyloliquefaciens*.

In conclusion, we have presented results which support the identification of the biosynthetic genes involved in DNJ biosynthesis, up to production of mannojirimycin. Ongoing studies include the identification of the remaining biosynthetic machinery required to convert mannojirimycin to deoxynojirimycin. As suggested in Scheme 1, this is possibly accomplished in two steps via epimerization and reduction, though alternate schemes may be proposed.[21] The region of the *B. amyloliquefaciens* genome flanking the *gabT1-yktC1-gutB1* cluster has no obvious candidates for these enzymatic activities, suggesting that they may be located elsewhere in the genome.

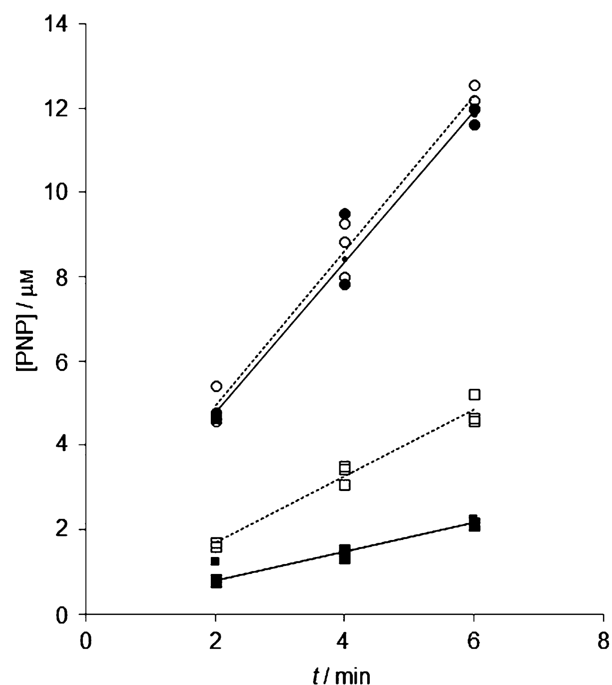

Figure 3. Detection of azasugar production in recombinant *E. coli*. *E. coli* culture media extracts for empty vector (circles) and *gabT1-yktC1-gutB1*-pETBlue-2 transformants (squares) were added to mannosidase assay mixtures to detect inhibition. Mannojirimycin was assayed from Dowex eluates of culture media (filled symbols) and deoxymannojirimycin from their $NaBD_4$ reduction products (open symbols). See text and the Supporting Information for further details.

Acknowledgements

This work was supported by the University of Florida and National Science Foundation (MCB-1020940). We thank Drs Valerie de Crecy-Lagard, Max Teplitski, Nigel Richards, and Daniel Zeigler for helpful discussions.

Keywords: azasugars · *Bacillus amyloliquefaciens* · biosynthesis · deoxynojirimycin · mannojirimycin

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[21] In principle, an alternate route between mannojirimycin and DNJ requiring only one enzyme could involve spontaneous dehydration to the imine, tautomerization to the β-hydroxyl enamine, tautomerization to the 2-keto isomer, and enzymatic reduction to DNJ.

Received: June 3, 2011
Published online on July 22, 2011

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