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MEETING PRESENTATION

The Glycine Lipids of *Bacteroides thetaiotaomicron* Are Important for Fitness during Growth *In Vivo* and *In Vitro*

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ABSTRACT Acylated amino acids function as important components of the cellular membrane in some bacteria. Biosynthesis is initiated by the *N*-acylation of the amino acid, and this is followed by subsequent *O*-acylation of the acylated molecule, resulting in the production of the mature diacylated amino acid lipid. In this study, we use both genetics and liquid chromatography-mass spectrometry (LC-MS) to characterize the biosynthesis and function of a diacylated glycine lipid (GL) species produced in *Bacteroides thetaiotaomicron*. We, and others, have previously reported the identification of a gene, named *glsB* in this study, that encodes an *N*-acyltransferase activity responsible for the production of a monoacylated glycine called *N*-acyl-3-hydroxypalmitoyl glycine (or commendamide). In all of the *Bacteroidales* genomes sequenced so far, the *glsB* gene is located immediately downstream from a gene, named *glsA*, that is also predicted to encode a protein with acyltransferase activity. We use LC-MS to show that the coexpression of *glsB* and *glsA* results in the production of GL in *Escherichia coli*. We constructed a deletion mutant of the *glsB* gene in *B. thetaiotaomicron*, and we confirm that *glsB* is required for the production of GL in *B. thetaiotaomicron*. Moreover, we show that *glsB* is important for the ability of *B. thetaiotaomicron* to adapt to stress and colonize the mammalian gut. Therefore, this report describes the genetic requirements for the biosynthesis of GL, a diacylated amino acid species that contributes to fitness in the human gut bacterium *B. thetaiotaomicron*.

IMPORTANCE The gut microbiome has an important role in both health and disease of the host. The mammalian gut microbiome is often dominated by bacteria from the *Bacteroidales*, an order that includes *Bacteroides* and *Prevotella*. In this study, we have identified an acylated amino acid, called glycine lipid, produced by *Bacteroides thetaiotaomicron*, a beneficial bacterium originally isolated from the human gut. In addition to identifying the genes required for the production of glycine lipids, we show that glycine lipids have an important role during the adaptation of *B. thetaiotaomicron* to a number of environmental stresses, including exposure to either bile or air. We also show that glycine lipids are important for the normal colonization of the murine gut by *B. thetaiotaomicron*. This work identifies glycine lipids as an important fitness determinant in *B. thetaiotaomicron* and therefore increases our understanding of the molecular mechanisms underpinning colonization of the mammalian gut by beneficial bacteria.

KEYWORDS glycine lipid, stress

Members of the phylum *Bacteroidetes*, including genera containing important human gut commensal bacteria such as *Bacteroides*, *Parabacteroides*, and *Prevotella*, dominate the healthy human gut microbiota (1). The gut-associated *Bacteroidetes* are required to digest complex dietary glycans into short-chain fatty acids (SCFA) (such as acetate and propionate) that are accessible to the host (2–4). A

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longitudinal study in infants revealed the presence of *Bacteroides* in the infant gut within 1 week of birth, and some species of *Bacteroides* have been shown to utilize the polysaccharides present in human breast milk (5, 6). Therefore, it has been suggested that *Bacteroides* may have an important role during the early development of the infant gut (6).

Acylated amino acids can be found in the membranes of many bacteria (7, 8). The best-characterized, and most widespread, acylated amino acid is ornithine lipid (OL). OL contains a 3′-hydroxy fatty acid group attached by an amide linkage to the α-amino group of ornithine, with a second fatty acid group ester linked to the 3′-hydroxy group of the first fatty acid (9). The genetics of OL production were first described for *Sinorhizobium meliloti*, where it was shown that an *N*-acyltransferase encoded by *olsB* catalyzed the attachment of a hydroxylated fatty acid group to ornithine, resulting in monoacylated ornithine or lyso-OL (10). The second fatty acid was subsequently attached to the hydroxyl group of lyso-OL through the activity of an *O*-acyltransferase encoded by *olsA*, resulting in the production of OL (11). An enzyme called OlsF, containing functionally independent domains with *N*-acyltransferase and *O*-acyltransferase activities, has recently been shown to produce OL in *Serratia proteamaculans* (12). Activities that further modify the OL by hydroxylation or methylation have also been identified (13–15). OL have been shown to be important for growth during acid and temperature stress in *Rhizobium tropici*, and depletion of OL results in an increase in the speed of crown gall tumor formation in plants infected with *Agrobacterium* (13, 16). Therefore, OL are important during the interactions between bacteria and their environment, including hosts.

We, and others, have previously identified a gene from *Bacteroides*, initially named *choA*, that encodes an *N*-acyltransferase required for the production of a monoacylated glycine species, called *N*-acyl-3-hydroxy-palmitoyl glycine or commendamide (17, 18). We have previously reported that *choA* encodes a protein with homology to the *N*-acyltransferase domain of OlsF, suggesting a potential role for *choA* in the production of a diacylated glycine lipid (GL) species (17). In this study, we use liquid-chromatography-mass spectrometry (LC-MS) to show that *choA* is required for the production of a diacylated GL in *Bacteroides thetaiotaomicron*, and in line with previous nomenclature, we have renamed *choA* as *glsB*. Using heterologous expression in *Escherichia coli*, we show that GL production also requires *glsA*, a gene predicted to encode an *O*-acyltransferase that is located immediately upstream from *glsB* in the *B. thetaiotaomicron* genome. Finally, we show that *glsB* is important for the ability of *B. thetaiotaomicron* to adapt to stress and colonize the mammalian gut.

(This article was submitted to an online preprint archive [19].)

RESULTS

ChoA and ChoB are required to produce diacylated GL in *E. coli*. We have previously shown that *choA*, when expressed in *E. coli*, results in the production of commendamide, an *N*-acylated (3-OH C16:0) derivative of glycine with hemolytic activity and the ability to solubilize cholesterol micelles (17). In all members of the *Bacteroidales*, the *choA* gene is located immediately downstream from another gene (nominally called *choB*) predicted to encode an *O*-acyltransferase (17). Together, *choA* and *choB* are homologous to the bifunctional amino acid acyltransferase OlsF that is responsible for the production of OL in *S. proteamaculans* (Fig. 1). Therefore, we wanted to determine whether *choA* and *choB* might work together to produce diacylated glycine lipids (GL). To do this, we amplified BVU_RSO7720 (*choA*), BVU_RS07715 (*choB*), and *choB-choA* from *Bacteroides vulgatus* and cloned the genes into pBAD24 for arabinose-controlled expression in *E. coli* (resulting in the formation of pBAD-*choA*, pBAD-*choB*, and pBAD-*choBA*, respectively). The cells were cultured in LB broth, and gene expression was induced by the addition of 0.2% (wt/vol) L-arabinose to the cultures (as described in Materials and Methods). Cells were harvested, and methanol extracts of the cell pellets were subjected to high-resolution LC-MS analysis. In cells overexpressing *choA*, we detected lipid

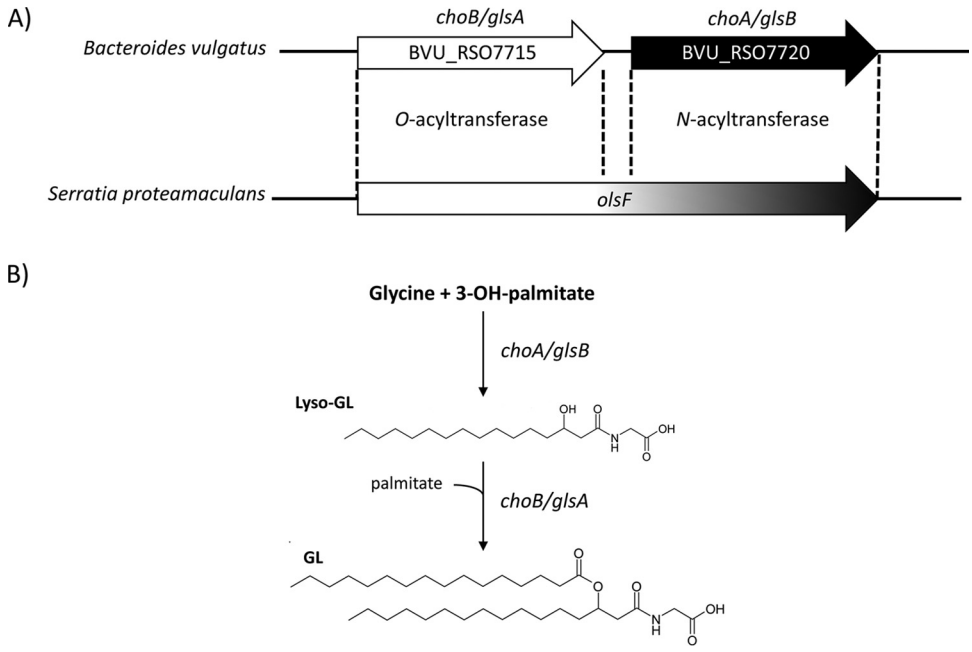

FIG 1 (A) The protein encoded by the *choB* gene in *Bacteroides vulgatus* has predicted homology (30.2% identity over 273 amino acids) with the N terminus of OlsF (Spro_2569), carrying the *O*-acyltransferase activity required for the biosynthesis of ornithine lipids (OL). Similarly, the *choA* gene is predicted to encode a protein with homology (32.6% identity over 264 amino acids) to the C terminus of OlsF, carrying the *N*-acyltransferase activity involved in OL biosynthesis. (B) Predicted pathway for the production of glycine lipid (GL) in *Bacteroides* based on homology with OL production in *Serratia proteamaculans*. In this schematic, the *N*- and *O*-acylations involve 3-OH-palmitate and palmitate, respectively.

species that corresponded to a glycine with an *N*-acyl substitution of various carbon chain lengths and degrees of saturation, primarily 14:0, 16:0 (i.e., commendamide), 16:1, and 18:1 (Fig. 2 and Table 1). Notably, overexpression of *choA* also resulted in the production of very low levels of diacylated glycine, i.e., 0.7% of the total acylated glycine pool (Table 1). When cells carrying pBAD-*choBA* were analyzed, we detected a range of both monoacylated and diacylated glycine species (Fig. 2 and Table 1). The identity of these lipids was confirmed by tandem MS (MS/MS) fragmentation, although some diacylated glycine species appeared to have mixed fatty acid compositions; e.g., in the peak with a mass-to-charge ratio (*m/z*) value of 510.4, there was a mixture of diacylated glycines substituted with 3-OH-16:0 + 12:0 and 3-OH-14:0 + 14:0 (3-OH-16:0 + 12:0 indicates a glycine that is substituted with 2 acyl chains, C16:0 and C12:0) (Table 1). The total level of monoacylated glycine production in cells overexpressing *choBA* was approximately 4.5-fold lower than the level observed in cells overexpressing *choA* alone. Moreover, diacylated glycine production in *choBA*-expressing cells accounted for 92.7% of the total acylated glycine pool (Table 1). Importantly, we could not detect monoacylated or diacylated glycine in cells overexpressing *choB* alone (Fig. 2). Therefore, we propose that, in a mechanism analogous to OL biosynthesis, ChoA *N*-acylates glycine, resulting in the formation of lyso-GL, which is subsequently *O*-acylated by ChoB to produce diacylated GL. In accordance with the nomenclature used for the genes involved in OL biosynthesis, we propose that *choA* and *choB* be renamed *glsB* (encoding glycine *N*-acyltransferase) and *glsA* (encoding lyso-GL *O*-acyltransferase), respectively (Fig. 1B).

GlsB is required for the production of commendamide in *B. thetaiotaomicron*. We wanted to confirm that *glsA* and *glsB* were involved in the production of GL in *Bacteroides*. To do this, we decided to take advantage of the genetic tools available from *Bacteroides thetaiotaomicron* VPI-5482 in order to make a deletion mutant of the *glsB* (BT_3459) homologue in this bacterium. We also constructed a strain whereby a

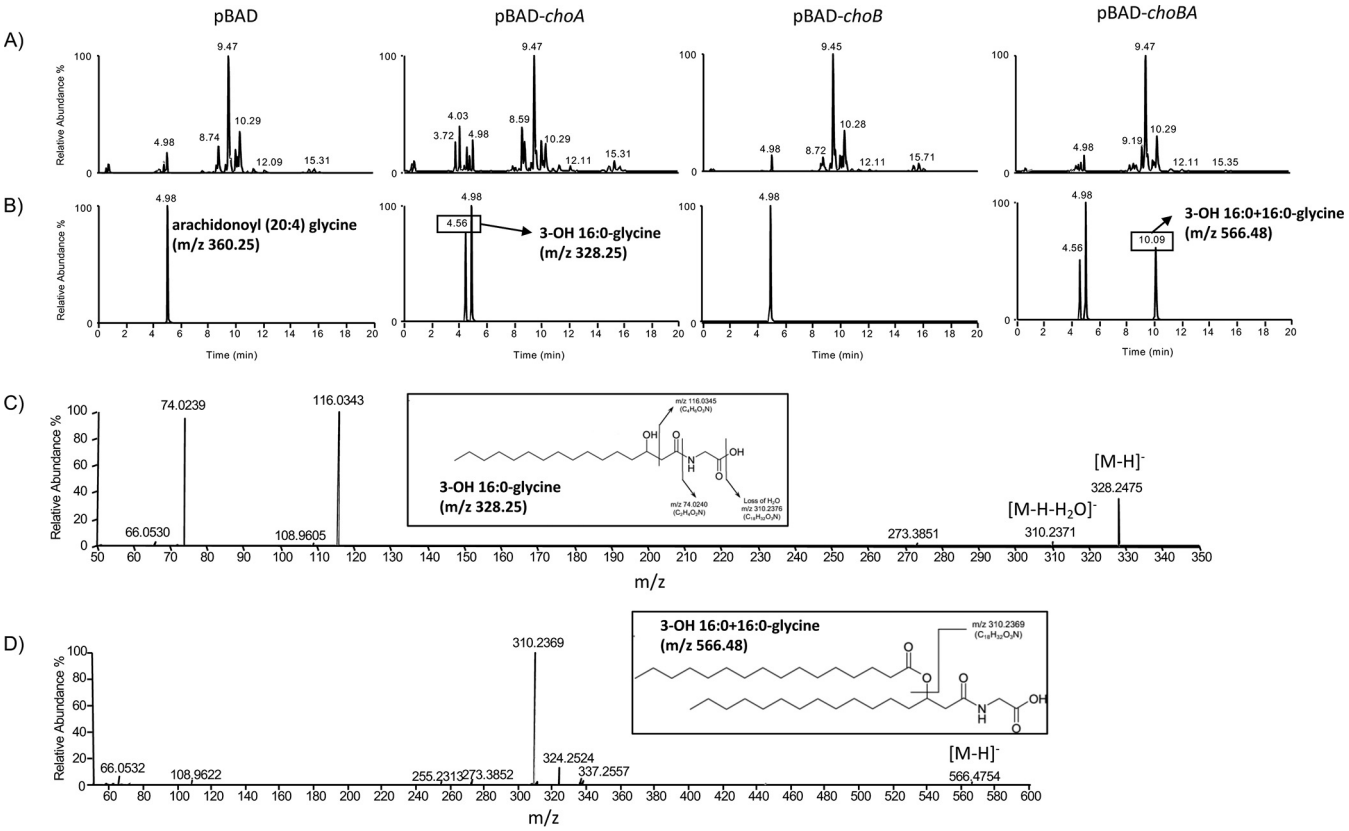

FIG 2 Identification of glycine lipids in *E. coli* overexpressing *choA* and *choB*. *E. coli* containing pBAD24, pBAD-*choA*, pBAD-*choB*, or pBAD-*choBA* was cultured in the presence of 0.2% L-arabinose until mid-exponential phase, and extracted lipids were analyzed by LC-MS, as described in Materials and Methods. All samples were spiked with 500 pmol arachidonyl (20:4) glycine as an internal standard (R_f = 4.98 min; *m/z* 360.25). (A) Base peak intensity chromatogram showing the range of lipids present in the *E. coli* membrane. All lipid profiles appear to be qualitatively similar, with the exception of that for pBAD-*choA*, which shows increased peaks eluting with an R_f of approximately 4 min, where monoacylated glycine molecules would be expected to be eluted. (B) Extracted ion chromatograms of peaks eluting with R_f values corresponding to mono- or diacylated glycine species. (For clarity, only 3-OH-16:0 [R_f = 4.56 min; *m/z* 328.25] and 3-OH-16:0 + 16:0 [R_f = 10.09 min; *m/z* 566.48] are shown. The full list of identified molecules can be found in Table 1.) (C) MS/MS fragmentation of the molecule eluting with an R_f of 4.56 confirming its identification as 3-OH-16:0-glycine. The same peak in both pBAD-*choA* and pBAD-*choBA* gave identical fragmentation profiles. (D) MS/MS fragmentation confirming the *O*-acylation of 3-OH-16:0 resulting in a structure of the *m/z* 566.48 compound that is consistent with a diacylated glycine (in this case, 3-OH-16:0 + 16:0). All diacylated glycine molecules detected are listed in Table 1.

native copy of *glsB* was inserted into the genome of the Δ*glsB* mutant strain (Δ*glsB*::*glsB*). Unfortunately, despite several attempts, we were unable to construct a knockout mutation of *glsA* (BT_3458), or a double knockout of *glsA glsB*, in *B. thetaiotaomicron*. Nonetheless, we were able to identify lipid species in both the wild type (WT) and the complemented Δ*glsB*::*glsB* strain that were consistent with commendamide and other *N*-acylated derivatives of glycine (Fig. 3 and Table 2). Further mass spectrometric analysis indicated the presence of diacylated GL in both the WT and the complemented Δ*glsB*::*glsB* strain but not in the Δ*glsB* mutant (Fig. 3B and Table 2). These species showed differences in their retention times compared to the GL found in *E. coli* and gave rise to multiple, partially resolved chromatographic peaks. *Bacteroides* strains are known to produce branched-chain fatty acids, although from our MS/MS analysis, it was not possible to definitively assign whether the glycines were acylated with straight- or branched-chain acyl groups (e.g., 16:0 or methyl-15:0) or if they were iso or anteiso branched (20). Importantly, we could not detect any acylated glycine in the Δ*glsB* mutant (Table 2). Therefore, *glsB* is required for the production of all acylated glycine species in *B. thetaiotaomicron*. Interestingly, a comparison of the total lipid chromatograms revealed both qualitative and quantitative differences between the Δ*glsB* mutant and both the WT and Δ*glsB*::*glsB* strains (Fig. 3A). Although a comprehensive analysis of these differences is not the objective of this study, we determined that an ion with the same mass as lipid 654 is produced by *B. thetaiotaomicron* but absent from the Δ*glsB*

TABLE 1 Glycine lipids in *E. coli* expressing *glsB* and *glsA*[c]

Predicted size of acyl group (*m/z*)	Mean concn (pmol/10^9 cells) ± SD	
	pBAD-*choA/glsB*	pBAD-*choBA/glsAB*
3-OH-12:0	35.3 ± 9.0	ND
3-OH-14:0 (300.2)[b]	4,070.3 ± 220.9	221.3 ± 145.2
3-OH-14:1 (298.2)[b]	118.0 ± 13.3	ND
3-OH-15:0 (314.2)[b]	52.4 ± 14.4	9.2[a]
3-OH-16:0 (328.2)[b]	3,954.6 ± 215.3	1,584.9 ± 843.4
3-OH-16:1 (326.2)[b]	6,831.9 ± 264.0	382.3 ± 267.1
3-OH-18:0 (356.1)[b]	7.8 ± 1.1	37.4 ± 33.7
3-OH-18:1 (354.2)[b]	2,539.2 ± 227.5	1,697.4 ± 994.2
Total monoacylated glycine	17,609.5 ± 965.5	3,923.3 ± 2,283.6
3-OH-14:0 + 12:0 (482.4)[b]	ND	436.6 ± 228.5
3-OH-16:0 + 12:0/3-OH-14:0 + 14:0 (510.4)[b]	ND	5,864.1 ± 2,999.9
3-OH-16:1 + 12:0 (508.4)[b]	ND	652.0 ± 356.9
3-OH-29:0	ND	240.5 ± 122.1
3-OH-16:0 + 14:0 (538.4)[b]	16.3 ± 5.7	17,407.0 ± 6,125.0
3-OH-16:1 + 14:0/3-OH-16:0 + 14:1 (536.4)[b]	ND	5,154.6 ± 2,257.9
3-OH-30:2	ND	452.0 ± 238.0
3-OH-16:0 + 15:0 (552.4)[b]	ND	458.8 ± 228.3
3-OH-31:1	ND	152.3 ± 47.2[a]
3-OH-16:0 + 16:0 (566.4)[b]	44.5 ± 10.8	3,901.3 ± 141.9
3-OH-16:0 + 16:1/3-OH-18:1 + 14:0 (564.4)[b]	39.4 ± 14.6	10,673.8 ± 3,157.3
3-OH-16:1 + 16:1 (562.4)[b]	ND	1,322.0 ± 429.6
3-OH-33:1	ND	131.4 ± 74.3
3-OH-16:0 + 18:1/3-OH-18:1 + 16:0 (592.5)[b]	27.7 ± 15.2	1,540.3 ± 23.7
3-OH-18:1 + 16:1 (590.4)[b]	ND	1,275.5 ± 218.8
3-OH-36:2	ND	179.5 ± 43.9
Total diacylated glycine	127.9 ± 46.3	49,841.7 ± 16,693.3
Total acylated pool	17,737.4	53,765.0

[a]Not detected in all biological replicates.
[b]Acylated glycine confirmed by MS/MS fragmentation. The acyl group designation is indicative, based on the predicted number of carbons in the lipid species.
[c]No acylated glycine molecules could be detected in cells carrying the pBAD vector alone. ND, not detected.

mutant (Fig. 3C). Lipid 654 is an acylated serine-glycine dipeptide that has been detected in many members of the *Bacteroidetes* (21). Lipid 654, also called flavolipin, was first described in members of the *Flavobacterium* and *Cytophaga* genera (22–24). Some recent studies with *Porphyromonas gingivalis* have implicated lipid 654 in osteoblast differentiation and atherosclerosis in humans and have also identified lipid 654 as a potential microbiome-associated biomarker for multiple sclerosis (21, 25–27). In addition, a series of molecules with retention times of approximately 14 to 16 min and *m/z* ratios ranging from 1,200 to 1,300 were also completely absent from the Δ*glsB* mutant. The identity of these species is under investigation. Therefore, our data confirm that *glsB* is required for the production of GL and lipid 654 in *B. thetaiotaomicron*, and a mutation in *glsB* results in significant qualitative and quantitative changes in the lipid profile of the membranes of this bacterium.

Expression of *glsB* and *glsA* is constitutive in *B. thetaiotaomicron*. We wanted to examine the expression levels of *glsA* and *glsB* in *B. thetaiotaomicron* during growth. Cells were cultured to mid-exponential phase in either rich (brain heart infusion-supplemented [BHIS]) or defined (defined minimal medium [DMM]) growth medium, and total RNA was isolated. Reverse transcription-PCR (RT-PCR) analysis suggested that *glsA* and *glsB* are on different transcripts in *B. thetaiotaomicron* (Fig. 4A). To determine the expression profile of *glsA* and *glsB*, we examined the data from previously reported microarray experiments undertaken using *B. thetaiotaomicron* cultured under different *in vitro* and *in vivo* conditions (28, 29). In TYG broth, a rich growth medium composed of tryptone, yeast extract, and glucose, the levels of expression of both *glsA* and *glsB* are highest during early exponential phase, and the expression of both genes decreases over time (Fig. 4B). A similar trend is observed during growth in minimal medium, but

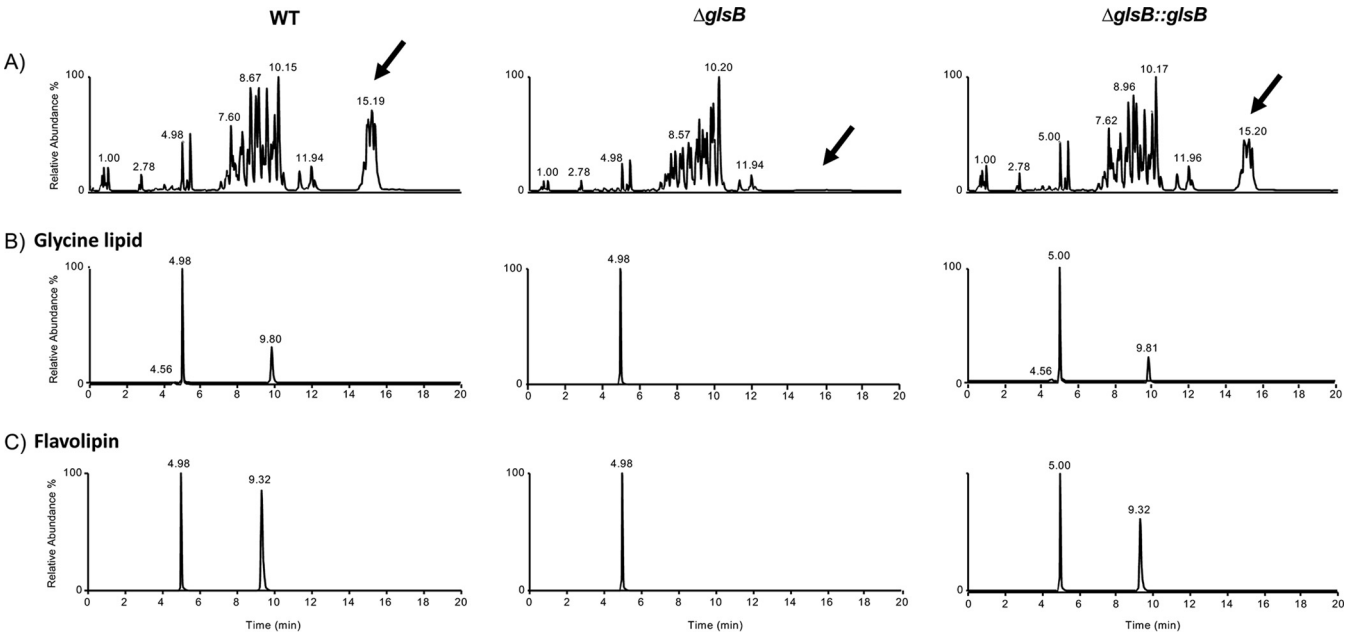

FIG 3 Identification of glycine lipids in *B. thetaiotaomicron*. Cells (as indicated) were cultured in BHIS broth until mid-exponential phase, and extracted lipids were analyzed by LC-MS, as described in Materials and Methods. All samples were spiked with 500 pmol arachidonyl (20:4) glycine as an internal standard (R_f = 4.98 min; *m/z* 360.25). (A) Base peak intensity chromatogram showing the range of lipids present in the membrane of *B. thetaiotaomicron*. Clear qualitative (indicated by arrows) and quantitative differences are observed between the profiles of the WT, Δ*glsB*, and Δ*glsB*::*glsB* strains. (B) Extracted ion chromatograms of peaks eluting with R_f values corresponding to mono- or diacylated glycine species. For clarity, the internal standard (arachidonoyl [20:4] glycine [R_f = 4.98 to 5.00 min; *m/z* 360.25]) and 2 ions corresponding to 3-OH-16:0 (R_f = 4.56 min; *m/z* 328.25) and $C_{32:0}$ (R_f = 9.8 min; *m/z* 566.4) are shown. The full list of identified molecules can be found in Table 2. (C) Extracted ion chromatograms of peaks eluting with R_f values corresponding to flavolipin (lipid 654). The internal standard and the ion corresponding to $C_{32:0}$ flavolipin (R_f = 9.32 min; *m/z* 653.51) are shown.

the decrease in *glsA* and *glsB* expression over time is not as strong (Fig. 4B). Therefore, the expression of *glsA* and *glsB* may be linked to the growth rate. Moreover, *glsA* and *glsB* are also expressed during colonization of the cecum of mice by *B. thetaiotaomicron* (Fig. 4B). Therefore, *glsA* and *glsB* appear to be constitutively expressed in *B. thetaiotaomicron* during growth *in vitro* and *in vivo*.

The *glsB* gene is required for normal growth *in vitro*. During preliminary experiments, we observed that when colonies were inoculated from agar plates into BHIS broth and incubated overnight, there was significantly reduced growth of the Δ*glsB* mutant compared to WT cultures. This suggested that *glsB* might be important for the normal growth of *B. thetaiotaomicron*. In order to quantify this observation, we set up

TABLE 2 Glycine lipids in *B. thetaiotaomicron*

Predicted size of acyl group (*m/z*)	Mean concn (pmol/10^9 cells) ± SD[c]		
	WT	Δ*glsB*	Δ*glsB*::*glsB*
3-OH-15:0/3-OH-methyl 14:0 (314.2)[b]	67.9 ± 23.8[a]	ND	64.6 ± 16.9[a]
3-OH-16:0/3-OH-methyl 15:0 (328.2)[b]	252.1 ± 36.3[a]	ND	137.2 ± 98.1
3-OH-17:0/3-OH-methyl 16:0 (342.2)[b]	429.9 ± 71.6[a]	ND	223.0 ± 157.0
Total monoacylated glycine	749.9 ± 131.7	0	424.8 ± 272
3-OH-methyl 15:0 + 13:0/3-OH-methyl 14:0 + 14:0 (524.4)[b]	223.2 ± 25.5	ND	169.4 ± 30.9
3-OH-methyl 15:0 + 14:0/3-OH-methyl 14:0 + 15:0 (538.4)[b]	792.1 ± 81.2	ND	635.4 ± 92.0
3-OH-methyl 15:0 + 15:0/3-OH-methyl 16:0 + 14:0 (552.4)[b]	2,000.3 ± 139.3	ND	1,372.0 ± 181.3
3-OH-methyl 16:0 + 15:0/3-OH-methyl 15:0 + 16:0 (566.4)[b]	2,602.6 ± 118.9	ND	1,768.0 ± 259.6
Total diacylated glycine	5,618.2 ± 364.9	0	3,944.8 ± 563.8
Total acylated pool	6,368.1	0	4,369.6

[a]Detected in only 2 (out of 3) biological replicates.
[b]Acylated glycine confirmed by MS/MS fragmentation. The acyl group designation is indicative, based on the predicted number of carbons in the lipid species.
[c]ND, not detected.

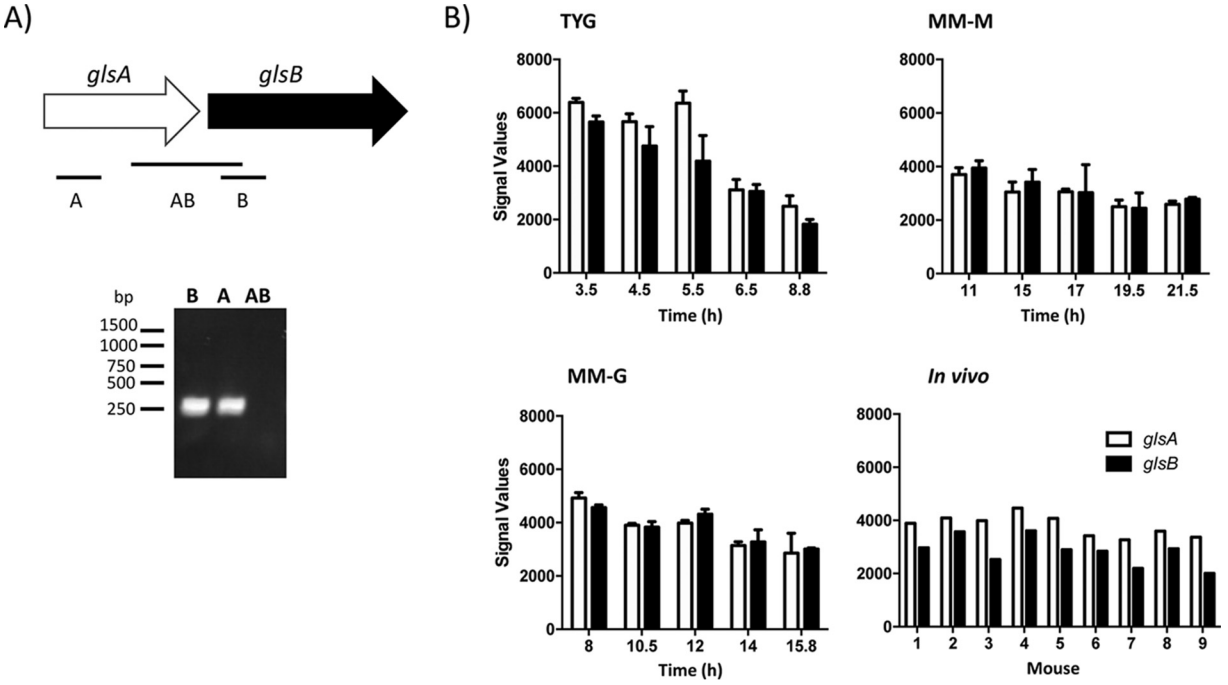

FIG 4 Analysis of the expression of *glsA* and *glsB* in *B. thetaiotaomicron*. (A) RT-PCR transcript analysis of expression from the *glsAB* locus. *B. thetaiotaomicron* cells were grown to mid-exponential phase in BHIS broth, and RNA was extracted and back-transcribed into cDNA (as described in Materials and Methods). Transcript analysis was undertaken using primer combinations that amplify a region specific to *glsA* (A) or *glsB* (B) or the intergenic region (AB). (B) Expression of *glsA* and *glsB* under different *in vitro* and *in vivo* growth conditions. Normalized microarray data were extracted from data sets available in the GEO database (accession number GSE2231). Sample preparation and analysis were described previously (28, 29). For the *in vitro* samples, *B. thetaiotaomicron* was cultured in chemostats using different media (TYG broth, minimal medium with maltose [MM-M], and minimal medium with glucose [MM-G]). At the indicated times, cells were harvested, RNA was extracted, and expression profiling was undertaken using custom *B. thetaiotaomicron* GeneChips. The data presented are the means of results from 2 biological replicates, and the error bars represent the standard deviations. For the *in vivo* experiments, individual germfree NMRI mice ($n = 9$) were monoassociated with *B. thetaiotaomicron* and fed a standard chow diet for 10 days. RNA was extracted from the cecal contents of each mouse and used for expression profiling using the custom *B. thetaiotaomicron* GeneChips.

10 cultures, from fresh BHIS agar plates inoculated with the WT, the Δ*glsB* mutant, or the Δ*glsB*::*glsB* strain, and the cultures were incubated at 37°C for 18 h, at which point the final optical density at 600 nm (OD_{600}) was taken as a measurement of growth. WT and Δ*glsB*::*glsB* cultures grown under these conditions reached mean OD_{600} values of 1.42 ± 0.042 and 1.25 ± 0.25, respectively (Fig. 5). However, the OD_{600} values of cultures inoculated with the Δ*glsB* mutant were significantly lower (0.36 ± 0.23; $P < 0.001$), confirming that the Δ*glsB* mutant has a strong growth defect under these conditions. Interestingly, when Δ*glsB* mutant cells from the broth cultures grown overnight were inoculated into fresh broth cultures, there was no observed defect in growth rates between the WT and the Δ*glsB* mutant (Fig. 5B). Therefore, our data suggest that *glsB* may be required to facilitate the adaptation of *B. thetaiotaomicron* to the transition from growth on a solid surface to growth in liquid broth.

The *glsB* gene is required for adaptation to stress *in vitro*. The transition from growth in solid medium to growth in liquid medium may represent a stress to the bacterium. Therefore, we decided to assess the sensitivity of the Δ*glsB* mutant to different stresses that would normally be encountered by *B. thetaiotaomicron*, i.e., bile stress and the presence of oxygen in air. *B. thetaiotaomicron* was cultured in BHIS broth to mid-exponential phase before the cells were transferred to fresh medium supplemented with 1% (wt/vol) porcine bile, and the cells were incubated, anaerobically, for a further 14 h. Under these conditions, WT and Δ*glsB*::*glsB* cultures reached final cell densities of 2.6×10^9 CFU ml^{-1} and 2.7×10^9 CFU ml^{-1}, respectively (Fig. 6A). These cell densities are only marginally lower than the cell densities achieved when cells are grown under the same conditions but in the absence of bile (2.98×10^9 CFU ml^{-1} and 2.9×10^9 CFU ml^{-1}, respectively), and this reflects the high level of bile tolerance

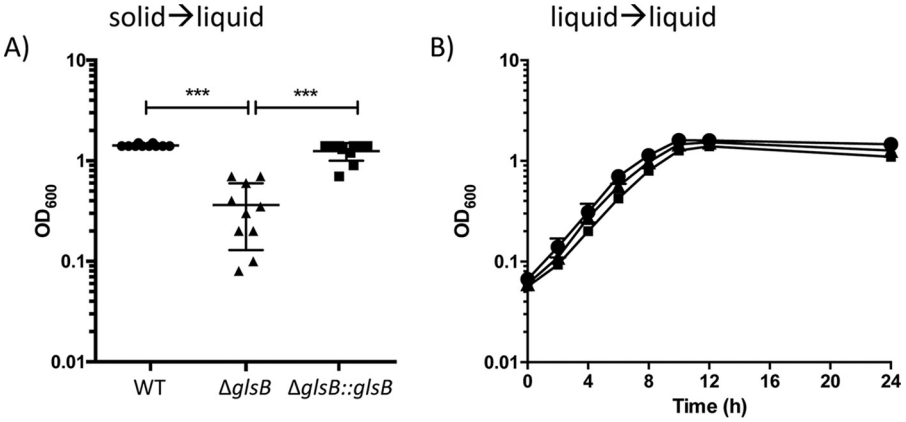

FIG 5 The Δ*glsB* mutant is unable to normally transition from solid to liquid growth media. (A) *B. thetaiotaomicron* (WT, Δ*glsB*, and Δ*glsB*::*glsB* strains) was grown on BHIS agar, and individual colonies ($n = 10$) were inoculated into fresh BHIS broth (solid→liquid). The cultures were incubated at 37°C for 24 h, and the OD_{600} was used to measure growth. Each point represents data for an individual culture, and the means (± standard deviations) are presented (***, $P < 0.0001$, as determined using one-way ANOVA with Tukey's posttest for multiple comparisons). (B) Cells cultured as described above for panel A were used to inoculate fresh BHIS broth, cells were grown at 37°C, and the OD_{600} was measured at the indicated times. Each strain was grown in triplicate, each point is the mean from the replicates, and errors bars represent the standard deviations.

associated with *Bacteroides* (30). In contrast, the Δ*glsB* mutant achieved a cell density of only 2.7×10^5 CFU ml^{-1} when cultured in the presence of 1% (wt/vol) porcine bile (in contrast to 2.7×10^9 CFU ml^{-1} when cultured in the absence of bile). Therefore, the Δ*glsB* mutant is approximately 10^4-fold more sensitive to porcine bile than the WT (Fig. 6A). Similarly, the Δ*glsB* mutant exhibited a 10-fold increased sensitivity to exposure to air for 14 h compared to both the WT and Δ*glsB*::*glsB* strains (Fig. 6B). Therefore, the *glsB* gene is important in *B. thetaiotaomicron* to allow adaptation to a variety of stresses, including exposure to bile and air.

The *glsB* gene is required for normal colonization of the murine gut. We wanted to determine the role, if any, of *glsB* during colonization of the mammalian gut. Therefore, germfree (GF) C57BL/6 mice were subjected to a single oral gavage of 10^8 CFU of either WT *B. thetaiotaomicron* or Δ*glsB* mutant bacteria. Fecal pellets were collected on days 2, 6, 9, and 12 postgavage, and bacteria were enumerated by viable plate counting on BHIS agar. On day 2, the level of WT *B. thetaiotaomicron* bacteria in fecal pellets was 3.6×10^{10} CFU g^{-1} feces, compared to a significantly lower level of

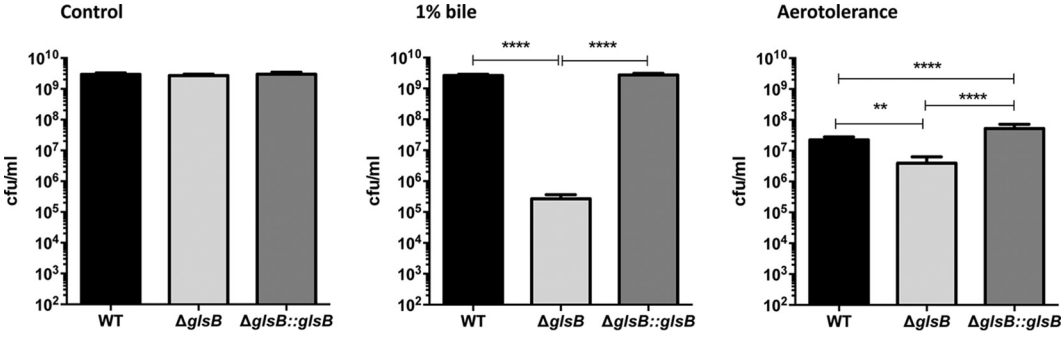

FIG 6 The *glsB* gene is required for adaptation to stress. *B. thetaiotaomicron* (WT, Δ*glsB*, and Δ*glsB*::*glsB* strains) was cultured to mid-exponential phase ($OD_{600} = 0.2$) before (i) inoculation into BHIS broth followed by anaerobic incubation for 14 h (control), (ii) inoculation into BHIS broth supplemented with 1% (wt/vol) porcine bile followed by anaerobic incubation for 14 h (1% bile), or (iii) inoculation into BHIS broth followed by aerobic incubation (with vigorous shaking) for 14 h (aerotolerance). Viable cell counts were determined using serial dilutions that were plated onto BHIS agar followed by anaerobic incubation for 24 to 48h. The experiment was repeated 3 times, and the error bars represent the standard deviations. Significance was determined using one-way ANOVA with Tukey's posttest for multiple comparisons (**, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

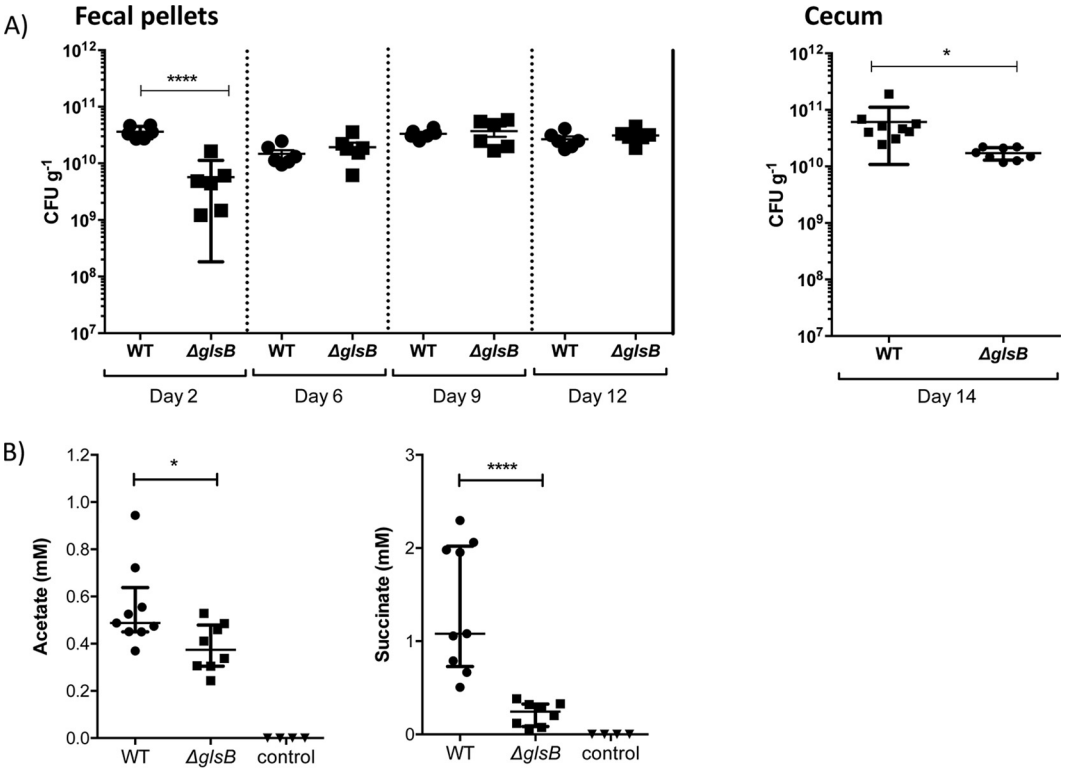

FIG 7 *B. thetaiotaomicron* requires the *glsB* gene for normal colonization of the gut of mice. Germfree C57BL/6 mice were gavaged with a single dose of 10^8 CFU of the *B. thetaiotaomicron* WT ($n = 9$) or Δ*glsB* mutant ($n = 8$) strain, as indicated. Control mice ($n = 4$) were not colonized. (A) At the indicated times postgavage, fecal pellets were collected, and bacteria were enumerated by plating fecal homogenates onto BHIS agar. Bacteria in the cecum of colonized mice were also enumerated on day 14 postgavage. Control mice did not contain any bacteria at this point. Error bars represent the standard deviations of colonization levels in at least 5 mice (*, $P < 0.05$; ****, $P < 0.001$ [by an unpaired *t* test]). (B) The contents of the cecum from each colonized and control mouse were examined for the presence of acetate and succinate by HPLC analysis. The error bars represent the 25th to 75th percentile values from the median, and significance was determined using the Mann-Whitney test (*, $P < 0.05$; ****, $P < 0.0001$).

5.7×10^9 CFU g^{-1} feces for the Δ*glsB* mutant (Fig. 7A). However, by day 4, the Δ*glsB* mutant was found to be present in fecal pellets at the same level as the WT. Analysis of the cecal contents of mice collected on day 14 indicated that there is a small, but significant, decrease in the level of the Δ*glsB* mutant in the cecum compared to WT *B. thetaiotaomicron* (Fig. 7B). Therefore, the Δ*glsB* mutant is affected in its ability to colonize the murine gut, particularly during the early stages of colonization.

An important role for *B. thetaiotaomicron* in the gut is the conversion of dietary glycans into SCFA such as acetate and other organic acids, e.g., succinate (4, 31). Therefore, we decided to use acetate and succinate production as markers of *B. thetaiotaomicron* metabolism in the host. Using high-performance liquid chromatography (HPLC), we measured the levels of acetate and succinate in the cecal contents collected from germfree mice infected with either the WT or the Δ*glsB* mutant. As expected, we could not detect any acetate or succinate in the cecal contents of uninfected mice, confirming that these metabolites are exclusively derived from microbial activity in the gut (Fig. 7C). There was a small, but significant ($P = 0.036$ by a Mann-Whitney test), decrease in the level of acetate present in the cecal contents of mice colonized with the Δ*glsB* mutant compared to the WT (0.37 mM [0.24 to 0.53 mM] versus 0.49 mM [0.37 to 0.94 mM], respectively). The level of succinate was also significantly ($P < 0.0001$ by a Mann-Whitney test) reduced in the cecal contents of mice infected with the Δ*glsB* mutant compared to WT bacteria (0.25 mM [0.06 to 0.38 mM] versus 1.1 mM [0.51 to 2.3 mM], respectively). Therefore, the metabolism of the Δ*glsB* mutant is different from that of WT *B. thetaiotaomicron* during growth in the murine gut.

DISCUSSION

The acylated amino acids GL and flavolipin have previously been identified in the membranes of several different members of the phylum *Bacteroidetes* (8, 21, 32). In this study, we have identified the genes required for the production of these acylated amino acids. Using genetics and high-resolution LC-MS, we show that *glsB* (BT_3459) encodes a glycine *N*-acyltransferase that is required for the production of both GL and flavolipin in *B. thetaiotaomicron*. We also present evidence that *glsA* (BT_3458), a gene found immediately upstream from *glsB* on the *B. thetaiotaomicron* genome, encodes an *O*-acyltransferase that is required for the efficient production of GL. Interestingly, the overproduction of *glsA* and *glsB* in *E. coli* results in the synthesis of only GL, suggesting the presence of another activity in *B. thetaiotaomicron* that may convert GL to flavolipin.

Flavolipin (i.e., lipid 654) has been reported to signal to eukaryotic cells through an interaction with Toll-like receptor 2 (TLR2) (27, 33). Diacylated lipid 654 has been shown to be converted into a more potent monoacylated derivative, lipid 430 (or lyso-lipid 654), through the action of phospholipase A2 activity in the host (21). *N*-Acyl amino acids are important endogenous signaling molecules produced in the human host; e.g., *N*-arachidonoyl glycine has been shown to block pain perception in mice (34–36). Genes encoding *N*-acyl synthases are enriched in the human gut microbiome, and both lipid 654 and lipid 430 have been found in tissues that are distal from the gut, indicating that these molecules can be distributed around the human host (21, 36). Therefore, there is accumulating evidence supporting an important role for the gut microbiota in the production of acylated amino acids, such as flavolipin, that can act as signaling molecules in the host.

During periods of phosphate starvation, some bacteria access cellular phosphate reserves by using acylated amino acids such as OL to replace the phospholipids normally found in bacterial membranes (37–39). On the other hand, OL production in *Rhizobium tropici* and *Burkholderia cepacia* has been shown to be important for the normal tolerance of the bacterial cell to acid and temperature stress (13, 40, 41). Our data suggest a role for GL/flavolipin during the response of *B. thetaiotaomicron* to different stresses, and a *glsB* deletion mutant was compromised in its ability to adapt to various stresses, including the transition from liquid to solid media, exposure to bile, and exposure to air. Interestingly, we could not construct a deletion in *glsA*, suggesting that this gene might be essential in *B. thetaiotaomicron*. However, IN-Seq analysis of *B. thetaiotaomicron* did not identify either *glsA* or *glsB* as an essential gene, although in the same study, both genes were identified as being important for normal growth *in vitro* (42). Therefore, it is clear that *glsA* and *glsB* have an important role in *B. thetaiotaomicron*. Interestingly, orthologues of *glsA* and *glsB* have been successfully deleted in *Bacteroides fragilis*, suggesting that the importance of *glsA* and *glsB* during growth might also be species dependent (43).

In this study, we show that the Δ*glsB* mutant in *B. thetaiotaomicron* is affected in its ability to colonize the gut of a GF mouse. A mutant with a deletion of both the *glsA* and *glsB* orthologues in *B. fragilis* (named *hlyB* and *hlyA*, respectively) was also attenuated for virulence in a mouse abscess model, supporting a role for these genes during *in vivo* growth (44). We have shown that, in contrast to the WT, the Δ*glsB* mutant produces decreased levels of both acetate and succinate while in the cecum, indicating that there are differences in metabolism between the WT and the Δ*glsB* mutant. Acetate and succinate are important end products of carbohydrate metabolism in *B. thetaiotaomicron*. Acetate is produced from acetyl-CoA via the acetate kinase (AckA)-phosphate acetyltransferase (Pta) pathway, resulting in the generation of ATP. The reduction in acetate production in the Δ*glsB* mutant is small and may not by physiologically important. Succinate production is via phosphoenolpyruvate, oxaloacetate, malate, and fumarate and is linked to the production of reducing equivalents [through the regeneration of $NAD(P)^+$] and the formation of a proton motive force. Fumarate reductase (Frd) catalyzes the production of succinate from fumarate, and this protein complex is localized to the inner membrane of *Bacteroides*. Therefore, one possible explanation for

TABLE 3 Strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
Strains		
Escherichia coli EPI300	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80d*lacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara leu*)*7697 galU galK* λ$^-$ *rpsL* (Str^r) *nupG trfA dhfr*	Epicentre
B. thetaiotaomicron	VPI 5482 with *tdk* deletion; Gm^r FUdR^r	Eric Martens, University of Michigan
B. thetaiotaomicron Δ*glsB*	Derivative of Δ*tdk* strain with *glsB* deletion; Gm^r FUdR^r	This study
B. thetaiotaomicron Δ*glsB*::*glsB*	Δ*glsB* strain with complementing *glsB* + 500 bp of upstream sequence; Gm^r Erm^r	This study
Plasmids		
pBAD24	Arabinose-inducible expression vector; Amp^r	47
pBAD24-*choA*	Arabinose-inducible expression vector; Amp^r	This study
pBAD24-*choB*	Arabinose-inducible expression vector; Amp^r	This study
pBAD24-*choBA*	Arabinose-inducible expression vector; Amp^r	This study
pEXCHANGE-Δ*tdk*	Carrying cloned *tdk*; Amp^r Erm^r	45
pEXCHANGE-Δ*tdk* Δ*glsB*	Carrying cloned *tdk*; Amp^r Erm^r	This study
pNbu2-bla-ermGb	Inserts into NBU2 att1 and/or att2 site; Erm^r Amp^r	45
pNbu2-bla-ermGb-*pglsB*	Chromosomal insertion vector; inserts into NBU2 att1 and/or att2 site; Erm^r Amp^r	This study

the reduced level of succinate observed in the *glsB* mutant of *B. thetaiotaomicron* is that the activity of Frd is compromised by the altered membrane composition of this mutant. Nonetheless, the significantly reduced levels of succinate produced by the Δ*glsB* mutant would be expected to compromise metabolic flux and, therefore, reduce fitness in the gut. In support of this, both *glsA* (BT_3458) and *glsB* (BT_3459) have been identified as important determinants for the colonization of the GF mouse gut during an IN-Seq screen with *B. thetaiotaomicron* (42). Therefore, we have shown that *glsB*, and presumably the production of GL and/or flavolipin, is an important fitness factor in *Bacteroides*, required for adaptation to stress and normal colonization of the mammalian gut, particularly in the presence of a competing microbiota.

MATERIALS AND METHODS

Strains, plasmids, primers, and growth conditions. *Bacteroides thetaiotaomicron* VPI 5482 was cultured anaerobically at 37°C in brain heart infusion medium (Sigma) supplemented with hemin (5 μg ml^{-1}), 0.1% (wt/vol) cysteine, and 0.2% (wt/vol) sodium bicarbonate. *Escherichia coli* EPI300 (Epicentre) was routinely cultured in LB broth at 37°C (Merck). For agar plates, 1.5% (wt/vol) agar was added to the liquid media. Where appropriate, the following antibiotics were added to the media: ampicillin (Amp) at 100 μg/ml, chloramphenicol (Cm) at 12.5 μg/ml, gentamicin (Gm) at 50 μg/ml or 200 μg/ml, and erythromycin (Ery) at 25 μg/ml. Plasmids and primers used in this study are shown in Tables 3 and 4.

Construction of gene knockouts in *B. thetaiotaomicron*. Gene deletions were carried out using *B. thetaiotaomicron* Δ*tdk*, as previously described (45). Briefly the DNA regions flanking the gene to be deleted were amplified and fused by PCR, cloned into the pEXCHANGE-*tdk* vector, and transformed into *E. coli* S17-1 λ*pir*. The donor (*E. coli*) and recipient (*B. thetaiotaomicron*) strains were mixed, plated onto

TABLE 4 Primers used in this study

Primer	Sequence (5′–3′)	Use in study
RTfwB300thet	ATACTTCGGACGAAAGCTCC	RT-PCR for *glsA* fragment from *B. thetaiotaomicron*
RTrvB300thet	CTTTACTTTCCCGTCATAATGG	RT-PCR for *glsA* fragment from *B. thetaiotaomicron*
RTfwA300thet	AAGAAATATTAGGTGGTTACCG	RT-PCR for *glsB* fragment from *B. thetaiotaomicron*
RTrvA300thet	GATAGCTGGGATACATAGTC	RT-PCR for *glsB* fragment from *B. thetaiotaomicron*
FwBA630thet	TTCCCAAGATGGTGGAAGCC	RT-PCR for *glsAB* fragment from *B. thetaiotaomicron*
RvBA630thet	TATTCGTCGATATCCATCGAC	RT-PCR for *glsAB* fragment from *B. thetaiotaomicron*
FwchoAFLANK1	CTGCTGGGATCCTCATCAGGACGAGATTAATG	*glsB* deletion from *B. thetaiotaomicron*
RvchoAFLANK1	GCACTCGATCTTTACCGGAAAATTACATATTCTTGTTATAGTGTTCTATC	*glsB* deletion from *B. thetaiotaomicron*
FwchoAFLANK2	GATAGAACACTATAACAAGAATATGTAATTTTCCGGTAAAGATCGAGTGC	*glsB* deletion from *B. thetaiotaomicron*
RvchoAFLANK2	CTGCTGTCTAGATACCCCTTTTCATCGAGCC	*glsB* deletion from *B. thetaiotaomicron*
FwchoAKOcheck	GGTTTCTTATCTGAAGAAAATAG	Checking *glsB* deletion in *B. thetaiotaomicron*
RvchoAKOcheck	TCAACGCTTGCCTCCATCG	Checking *glsB* deletion in *B. thetaiotaomicron*

BHIS agar containing 10% (vol/vol) horse blood (BHIS blood agar), and incubated aerobically at 37°C for 24 h. The biomass was resuspended in 5 ml TYG broth, before plating onto BHIS blood agar supplemented with Gm and Ery. The plates were incubated anaerobically for 48 h at 37°C, before 5 to 10 colonies were restreaked onto BHIS blood agar (Gm Ery). After 48 h at 37°C, single colonies were picked into TYG broth and grown for 20 h without antibiotics before plating onto BHIS blood agar supplemented with 200 μg ml^{-1} 5-fluoro-2′-deoxyuridine (FUdR) for vector counterselection. The plates were incubated anaerobically for 72 h and restreaked onto BHIS blood agar plates containing FUdR. Colony PCR, using primers that were designed outside the flanking regions, was used to identify potential knockout mutants, before confirmation by sequencing. For complementation experiments, the *glsB* (*choA*) gene, plus 500 bp upstream from the proposed translation start site, was amplified and cloned into the pNbu2-bla-ermGb insertion vector. The cloned DNA fragment was inserted into the *B. thetaiotaomicron* Δ*tdk* Δ*glsB* genome in either of the two Nbu2-targeted tRNA^{Ser} loci, via conjugation from *E. coli* S17-1 λ*pir*. The resulting complemented strain, *B. thetaiotaomicron* Δ*tdk* Δ*glsB*::*glsB*, was selected by plating onto BHIS blood agar supplemented with Gm and Ery, and the presence of the *glsB* gene was confirmed by PCR.

Colonization of germfree C57BL/6NTac mice. All experiments involving animals were performed at the Biological Services Unit at University College Cork and were approved by the University College Cork Animal Experimentation Ethics Committee. For colonization experiments, 6-week-old germfree female C57BL/6NTac mice were gavaged with 20 μl of 5×10^9 CFU ml^{-1} of the appropriate bacterial strain ($n = 9$ for WT *B. thetaiotaomicron*, $n = 8$ for *B. thetaiotaomicron* Δ*glsB*, and $n = 4$ for the uninoculated control). The mice were housed in groups of 2 to 3 in individually ventilated cages (IVC), and bacterial enumerations were carried out by serial dilution and plating of homogenized fecal pellets collected from each IVC on days 2, 6, 9, and 12 postgavage. All mice were euthanized on day 14, the ceca were harvested, and cecal contents were collected for further analysis, including bacterial enumeration.

Analysis of short-chain fatty acids. The level of short-chain fatty acids (SCFA) in the cecal contents was determined by HPLC using a protocol described previously (46). Cecal contents were weighed and resuspended in sterile MilliQ water (1:10 [wt/vol]) containing several 3- to 4-mm sterile glass beads (Sigma). The samples were vortexed for 1 min, and homogenates were centrifuged at 10,000 $\times$ *g* for 10 min. The supernatants were filter sterilized using a 0.22-μm filter and analyzed using HPLC with a refractive index detector (Agilent 1200 HPLC system). A Rezex 8μ, 8% H, 300- by 7.8-mm organic acid column (Phenomenex, USA) was used with 0.01 N H_2SO_4 as the elution fluid, at a flow rate of 0.6 ml min^{-1}. The temperature of the column was maintained at 65°C, and 20 μl of each sample was injected for analysis. End product peaks were identified by comparison of their retention times with those of pure compounds, and concentrations were determined from standards of known concentrations.

Identification and quantification of glycine lipids. Cultures of *E. coli*, with the appropriate plasmids, or *B. thetaiotaomicron* grown overnight were inoculated into fresh medium (LB broth with 0.2% [wt/vol] L-arabinose for *E. coli* or BHIS broth for *B. thetaiotaomicron*) at an OD_{600} of 0.05 and allowed to grow at 37°C until the OD_{600} reached 0.5 to 0.6. At this point, 1-ml samples were centrifuged (5 min at 12,000 $\times$ *g*), the pellets were resuspended in HPLC-grade methanol (Sigma), and 500 pmol *N*-arachidonyl glycine (NAGly) (20:4) (Cayman Chemicals, Ann Arbor, MI, USA) was added as an internal standard (ISTD). Ethyl acetate was added, and the mixture was left at 4°C for 30 min before being centrifuged at 2,000 $\times$ *g* for 5 min to remove denatured proteins. The supernatant was collected, evaporated to dryness under nitrogen gas, and reconstituted in methanol containing 5 mM ammonium formate (Sigma). LC-MS analyses were performed using a Thermo Exactive Orbitrap mass spectrometer (Thermo Scientific, Hemel Hempsted, UK) equipped with a heated electrospray ionization (HESI) probe and coupled to a Thermo Accela 1250 ultra-high-pressure liquid chromatography (UHPLC) system. Samples were injected onto a Thermo Hypersil gold C_{18} column (2.1 mm by 100 mm; 1.9 μm) maintained at 50°C. Mobile phase A consisted of water containing 10 mM ammonium formate and 0.1% (vol/vol) formic acid. Mobile phase B consisted of a 90:10 mixture of isopropanol-acetonitrile containing 10 mM ammonium formate and 0.1% (vol/vol) formic acid. The initial conditions for analysis were 65% mobile phase A–35% mobile phase B, and the percentage of mobile phase B was increased from 35% to 65% over 4 min, followed by 65% to 100% over 15 min, with a hold for 2 min before reequilibration to the starting conditions over 6 min. The flow rate was 400 μl/min. Samples were analyzed in negative-ion mode over the mass-to-charge ratio (*m/z*) range of 250 to 2,000 at a resolution of 100,000. The signals corresponding to the accurate *m/z* values for [M − H] ions of glycine lipid molecular species were extracted from raw LC-MS data sets with the mass error set to 5 ppm. Quantification was achieved by relating the peak area of the glycine lipid species to the peak area of the NAGly (20:4) ISTD. Tandem mass spectrometry (MS/MS) was employed to confirm the identity of glycine lipid species. Samples were infused at a rate of 5 μl/min into a Thermo LTQ-Orbitrap XL mass spectrometer and subjected to higher-energy collision dissociation (HCD) in the Orbitrap analyzer. Additional MS^3 analyses were performed through collision-induced dissociation (CID) in the ion trap. Collision energies ranged from 40 to 65%, and helium was used as the collision gas.

Reverse transcription-PCR. *Bacteroides* cultures grown overnight were subcultured into fresh BHIS broth to an OD_{600} of 0.05 and incubated anaerobically at 37°C until the cultures reached mid-exponential phase ($OD_{600} = 0.3$ to 0.5). At this stage, a 5-ml aliquot was removed and centrifuged, and the cell pellet was resuspended in RNAprotect (Qiagen). RNA extractions were carried out using a High Pure RNA isolation kit (Roche), according to the manufacturer's instructions. For the qualitative determination of gene expression, RNA was reverse transcribed into cDNA using a QuantiTect reverse transcription kit (Qiagen), according to the manufacturer's instructions. This cDNA was subsequently used as a template for PCR using 0.5 μl DNA template, 2.5 μl CoralLoad PCR buffer (Qiagen), 100 pmol of the appropriate primers (Table 4), 0.2 mM deoxynucleoside triphosphate (dNTP) mix (Promega), 0.125 μl *Taq* DNA

polymerase (Qiagen), and sterile MilliQ distilled water (dH_2O), in a final volume of 25 μl. The following PCR conditions were used: 95°C for 5 min (initial denaturation), followed by 35 cycles of 95°C for 30 s, the primer-specific annealing temperature for 30 s, and 72°C for the template-specific length of time (1 min per 1 kbp DNA). This was followed by a final extension step at 72°C for 10 min.

Stress assays. Cultures of the appropriate *B. thetaiotaomicron* strains grown overnight were adjusted to an OD_{600} of 0.05 in fresh BHIS broth and incubated for 7 h at 37°C anaerobically. At this point, the OD_{600} was adjusted to 0.2, and each culture was split into three equal aliquots, whereby one aliquot was cultured anaerobically, another aliquot was exposed to air, and the final aliquot was incubated in BHIS broth with 1% (wt/vol) porcine bile (Sigma). Following incubation for 14 h under the appropriate stress conditions, viable cells were enumerated by serial dilutions and plating onto BHIS agar.

Statistical analysis. All statistical analysis was performed using GraphPad Prism 6.0e for Mac software. All experiments were carried out using biological triplicate samples, unless stated otherwise. Student's *t* test or a Mann-Whitney test was used to compare two different groups of data, as indicated. One-way analysis of variance (ANOVA), with Tukey's posttest, was used to compare three or more groups of data, and differences were considered to be significant if the *P* value was <0.05.

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D.J.C. conceived the study, and A.L. carried out all of the experiments except for the lipidomics. P.D.W., S.R.T., and M.K.D. carried out all lipidomic experiments and analyses. A.L. and D.J.C. analyzed the data, and D.J.C. wrote the manuscript with the help of A.L. and P.D.W. All authors reviewed the manuscript.

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