JOURNAL OF BACTERIOLOGY, Aug. 1996, p. 4787–4793 Vol. 178, No. 16
0021-9193/96/$04.00+0

Identification and Functional Differentiation of Two Type I Fatty Acid Synthases in *Brevibacterium ammoniagenes*

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Received 10 April 1996/Accepted 6 June 1996

The fatty acid synthase (FAS) from *Brevibacterium ammoniagenes* is a homohexameric multienzyme complex that catalyzes the synthesis of both saturated and unsaturated fatty acids. By immunological screening of a *B. ammoniagenes* expression library, an *fas* DNA fragment was isolated and subsequently used to clone the entire gene together with its flanking sequences. Within 10,525 bp of sequenced DNA, the 9,189-bp FAS coding region was identified, corresponding to a protein of 3,063 amino acids with a molecular mass of 324,910 Da. This gene (*fasA*) encodes, at its 5′ end, the same amino acid sequence as is observed with purified *B. ammoniagenes* FAS. A second reading frame encoding another *B. ammoniagenes* FAS variant (FasB) had been identified previously. Both sequences are colinear and exhibit 61 and 47% identity at the DNA and protein levels, respectively. By using specific antibodies raised against a unique peptide sequence of FasB, this enzyme was shown to represent only 5 to 10% of the cellular FAS protein. Insertional inactivation of the FasB coding sequence causes no defective phenotype, while *fasA* disruptants require oleic acid for growth. Correspondingly, oleate-dependent *B. ammoniagenes* cells obtained by ethyl methanesulfonate mutagenesis were complemented by transformation with *fasA* DNA but not with *fasB* DNA. The data indicate that *B. ammoniagenes* contains two related though differently expressed type I FASs. FasA represents the bulk of cellular FAS protein and catalyzes the synthesis of both saturated and unsaturated fatty acids, while the minor variant, FasB, cannot catalyze the synthesis of oleic acid.

Unlike the majority of procaryotes, the coryneform bacterium *Brevibacterium ammoniagenes* contains an aggregated type I fatty acid synthase (FAS) multienzyme complex (5, 6). Apart from *B. ammoniagenes*, FAS proteins with this structural organization have also been found within the genera *Mycobacterium* (7, 26) and *Corynebacterium* (1). The taxonomic relatedness of *B. ammoniagenes* to corynebacteria in particular had already been proposed earlier, mainly on the basis of the occurrence of mycolic acid as a common constituent of their cell walls (24). In type I FASs, at least eight functionally different catalytic domains are integrated into either a single (bacteria, animals) or two different (fungi) multifunctional proteins (13). According to the pioneering work of Kawaguchi and coworkers (17), the *B. ammoniagenes* FAS complex is an α_6 homomultimer with a molecular mass of about 2.0 MDa. In contrast to the eucaryotic FAS enzymes, but like the dissociated type II bacterial FAS systems (3, 14), the *B. ammoniagenes* type I FAS contains a 3-hydroxydecanoyl-β,γ-dehydratase as an additional component; thus, both saturated and unsaturated fatty acids are synthesized by this enzyme (5). In a first attempt to investigate the molecular structure of this exceptional multifunctional FAS protein in more detail, we recently isolated and sequenced a 9,312-bp *fas*-like reading frame from *B. ammoniagenes* (16). The gene product encoded by this DNA exhibited 46% sequence similarity to yeast FAS, and its order of catalytic domains was colinear to a hypothetical head-to-tail fusion of the two yeast FAS subunits (16). However, disruption of this *fas*-like reading frame produced no FAS-defective *B. ammoniagenes* mutants, indicating that either it is not an FAS coding gene of *B. ammoniagenes* at all or it is not the only one. As will be reported in this paper, a continued immunological search indeed resulted in the isolation of a second *B. ammoniagenes* *fas* gene. Unlike the previously isolated *fas*-like DNA, the coding region for the N-terminal amino acid sequence of purified *B. ammoniagenes* FAS corresponded to this newly isolated coding sequence. On the basis of the observation that, in vivo, both *B. ammoniagenes* *fas* genes are expressed but that disruption of only one of them produces a fatty acid-requiring phenotype, the differential functions of the two genes are discussed.

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MATERIALS AND METHODS

Bacterial strains, culture conditions, and DNA isolation. *Escherichia coli* DH5α, Y1090r, and XL1 Blue MRA(P2), used for screening and amplification of recombinant phage and plasmid vectors, were purchased from Gibco/BRL (DH5α) and Stratagene, respectively. Other bacterial strains and the plasmids used for *B. ammoniagenes* transformation are listed in Table 1. *B. ammoniagenes* cells were grown at 30°C either in Luria broth (18) or in GCY medium (containing [per liter] 10 g of glucose, 10 g of Casamino Acids, 10 g of yeast extract [Gibco], and 3 g of NaCl), pH 7.3. Whenever indicated, media contained, in addition, kanamycin (50 or 20 mg/l). The lower antibiotic concentration was used when selecting for *B. ammoniagenes* conjugants, while the higher one served to ensure the stable propagation of transformants. Fatty acid-requiring mutants were supplemented with 0.03% oleic acid in the presence of 1% Tween 40. Alternatively, Brij 58 may have been used as a detergent.

Chromosomal DNA was isolated from wild-type and mutant *B. ammoniagenes* cells essentially as described by Meurer et al. (16) except that there was an additional incubation with RNase A (50 μg/ml) during cell lysis followed by an intensified proteinase K treatment (300 instead of 50 μg/ml). For plasmid isolation, *B. ammoniagenes* cells were suspended in TE buffer (50 mM Tris-HCl–10 mM EDTA, pH 8.0) and incubated with lysozyme (5 mg/ml) for 1 h at 37°C. Otherwise, the method of Birnboim and Doly (2) was followed. If necessary, plasmids were further purified on Quiagen ion-exchange columns (Diagen) according to the recommendations of the manufacturer.

DNA manipulation and computational techniques. Standard molecular biological techniques, such as PCR and Southern hybridization experiments, immunological screening for recombinants, and plasmid manipulations, were performed as described elsewhere (18). For DNA sequencing by the dideoxy chain

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Essential properties[a]	Reference
E. coli		
S 17-1	Mobilizing donor strain; MM 294 *recA*, with an RP4 derivative integrated into the chromosome	25
B. ammoniagenes		
ATCC 6871	Wild type, Nx^r	
BA 15-04	Oleic acid auxotroph obtained after EMS mutagenesis of ATCC 6871	This work
D1	Nx^r Km^r mutant due to integration of pECMΔSHM into the chromosomal *fasB* gene	This work
D2	Nx^r Km^r oleic acid auxotroph due to integration of pDV2 into the chromosomal *fasA* gene	This work
Plasmids		
pGH1	pECM1 containing the *fasB* gene together with 0.5 kb of both its 5′ and 3′ flanking regions	This work
pHPS2	pECM1 containing the *fasA* gene together with 3 kb of its 5′ and 1 kb of its 3′ flanking regions	This work
pECM1	Mobilizable *E. coli-B. ammoniagenes* shuttle vector; Km^r Cm^r	20
pECMΔ	*B. ammoniagenes* suicide vector derived from pECM1 by elimination of a 2.4-kb *Sph*I fragment	This work
pECMΔSHM	pECMΔ with an insertion of a central 1.5-kb *Sph*I-*Hin*dIII fragment derived from the *fasB* gene	This work
pDV2	pECMΔ with an insertion of the central *Eco*RI fragment (BF1) derived from the *fasA* gene	This work

[a] Abbreviations: Km^r, kanamycin resistance; Nx^r, nalidixic acid resistance; Cm^r, chloramphenicol resistance.

termination method of Sanger et al. (19), [α-^{35}S]-dATP was used along with the T7 Sequencing Kit from Pharmacia. Derivatives of plasmids pUC18 and pUC19 were used as sequencing templates throughout this work (18). Long-distance sequencing was performed by primer walking. Whenever necessary, sequence anomalies were resolved using 7-deaza-dGTP/dATP (Pharmacia) instead of dGTP/dATP. Sequencing primers were purchased from MWG Biotech (Ebersberg, Germany). The *B. ammoniagenes* lambda gt11 expression library and an EMBL3 library were constructed as described by Meurer et al. (16). Analysis of DNA and protein sequence data was done using the University of Wisconsin Genetics Computer Group sequence analysis software package, version 7.1. Sequence alignments were performed with the BestFit or PileUp programs using standard parameters.

Transformation of *B. ammoniagenes* cells. Conjugational plasmid transfer from *E. coli* S 17-1 (25) to *B. ammoniagenes* wild-type and mutant cells was performed according to the method of Schäfer et al. (20). All plasmids used for *B. ammoniagenes* transformation were derivatives of the vector pECM1 (20). For electroporation, cells were grown to mid-log phase and washed five times with cold 10% glycerol. At this stage, cells could be stored at −70°C. To obtain good yields of transformants, it was necessary to amplify the plasmid DNA in *B. ammoniagenes* rather than in *E. coli* cells. Approximately 0.5 μg of plasmid DNA was used for electroporation in a Bio-Rad Gene Pulser at 200 Ω, 25 μF, and 1.6 kV using an electroporation cuvette with a light path of 0.1 cm. The time constants thereby obtained were between 2.5 and 3.2 ms. After electroporation, cells were immediately incubated with ice-cold GCY medium containing 50 mM $CaCl_2$ and 50 mM $MgSO_4$ and placed on ice for 15 min. For cell regeneration, the suspension was diluted with standard GCY medium and incubated for 6 h at 30°C before being plated on kanamycin-containing selective media.

FAS purification and assays. The FAS purification procedure used for these studies was that originally described by Lynen (12) for the yeast FAS complex, with the following modifications. Frozen *B. ammoniagenes* cells (100 g) were suspended in 200 ml of 0.4 M potassium phosphate buffer, pH 7.5, containing 2 mM dithiothreitol. For inhibition of proteases, 100 mg of phenylmethanesulfonate (dissolved in 1 ml of acetone) was added. Cells were disrupted with glass beads at 4°C. The homogenate was fractionated by addition of ammonium sulfate, first to 30% and subsequently to 65% saturation. The 65% precipitate was dissolved in 0.1 M potassium phosphate, pH 7.5, and the FAS complex was sedimented by ultracentrifugation (100,000 × *g* for 10 h). The precipitate was dissolved in about 12 ml of 0.4 M potassium phosphate, pH 7.3, containing 2 mM dithiothreitol, and the resulting solution was then subjected to a 3-h centrifugation in a linear 5 to 25% sucrose density gradient at 185,000 × *g* in a Sorvall TV850 rotor. The FAS-containing fractions were collected, and the FAS complex was precipitated by addition of ammonium sulfate to 70% saturation. The precipitate was dialysed against 0.1 M potassium phosphate, pH 7.3, for 3 h. Subsequently, a second 5 to 25% sucrose density gradient centrifugation was performed for 20 h at 85,000 × *g* in a Beckman SW28 rotor. From the FAS-containing fractions, the complex was again sedimented by a 10-h centrifugation at 100,000 × *g*. Finally, the enzyme was dissolved in 0.1 M potassium phosphate buffer, pH 7.3, and subjected to fast protein liquid chromatography on a Superose 6 HR 10/30 gel filtration column (Pharmacia). The purity of the resulting FAS preparation was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (9). The acrylamide concentration was 3% for the stacking gel and 5% for the running gel. FAS activity was assayed according to the method of Lynen (12).

Immunological techniques. The production of a polyclonal antiserum against the purified *B. ammoniagenes* FAS was described previously (16). Domain-specific antisera against synthetic *B. ammoniagenes* FAS peptides were prepared by coupling them to keyhole limpet hemocyanin essentially as described by Liu et al. (11). FAS-specific peptides were synthesized by D. Palm (University of Würzburg, Würzburg, Germany). The keyhole limpet hemocyanin protein and the coupling reagent, 3-maleimidobenzoyl-*N*-hydroxysuccinimide ester, were purchased from Boehringer GmbH (Mannheim, Germany).

Western blotting (immunoblotting) was performed as described previously (21).

Nucleotide sequence accession numbers. The *B. ammoniagenes fasA* sequence reported in this paper has been submitted to the EMBL data bank and was assigned the accession number X 87822. The previously sequenced *fasB* gene has the accession number X 64795.

RESULTS

Purification of *B. ammoniagenes* FAS. The *B. ammoniagenes* FAS was isolated by a refined purification protocol relying mostly on separation methods based on the extraordinarily large molecular mass of this enzyme. The purified FAS protein thereby obtained proved to be homogeneous upon SDS-PAGE. By N-terminal sequencing, the 21-amino-acid sequence SLTPLHTLSNDSTAPAVLFAG was clearly established. This sequence was obviously different from the N-terminus sequence deduced from the previously isolated *B. ammoniagenes fas* gene. Therefore, the product of the gene

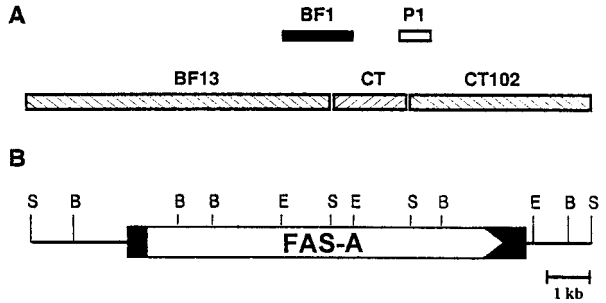

FIG. 1. Physical and restriction maps of the cloned *B. ammoniagenes* DNA region. (A) Compilation of cloned DNA fragments representing the original lambda gt11 clone BF1 (dark rectangle) and three subclones (BF13, CT, and CT102) derived from phages obtained by plaque hybridization of an EMBL3 library with BF1. Fragment P1 was generated from genomic *B. ammoniagenes* DNA by PCR amplification in order to verify the orientation of CT102. (B) Restriction map of the DNA region cloned in this work. The sequenced area is indicated by the bar. The FAS coding sequence therein is represented by the open arrow. Abbreviations: S, *Sal*I; B, *Bam*HI; E, *Eco*RI.

described by Meurer et al. (16) was unlikely to represent the main FAS in *B. ammoniagenes* cells.

Cloning and sequencing of a second *B. ammoniagenes fas* gene. In a first series of experiments, a lambda gt11 expression library of *Eco*RI-digested *B. ammoniagenes* DNA was screened with a polyclonal antiserum raised against purified *B. ammoniagenes* FAS. Among a total of 70,000 plaques investigated, 21 strongly cross-reacting phage clones were isolated, and their reproducibly immunopositive response was established. By restriction and Southern hybridization analyses, it was shown that all clones contained the same 1.8-kb DNA insert. This DNA fragment, BF1 (Fig. 1), was sequenced and proved to be free of stop codons in one of its reading frames. The deduced amino acid sequence exhibited significant similarity to the malonyl-palmitoyl transferase domain of the *Saccharomyces cerevisiae* FAS. As is evident from Fig. 2, this newly isolated FAS sequence (positions 1473 to 2078) was not identical to the corresponding region of the previously isolated *B. ammoniagenes fas* gene, although there were distinctive similarities. Thus, the BF1 DNA fragment was considered to be part of the second *B. ammoniagenes fas* gene that had been sought.

In order to isolate the N- and C-terminal flanking regions of BF1, an EMBL3 library containing *B. ammoniagenes* DNA partially digested with *Sau*3A was screened by plaque hybridization with BF1. Three phage clones were thereby isolated, with overlapping inserts of 14.2, 17, and 16.2 kb (data not shown). From these three clones, the plasmid subclones pBF13, pCT, and pCT102 were derived, covering about 16 kb of contiguous DNA on the *B. ammoniagenes* genome (Fig. 1). Within this DNA, a segment 10,525 bp in length was sequenced in both directions (Fig. 1). The sequenced DNA contains a 9,189-bp open reading frame with coding capacity for a protein of 3,063 amino acids with a molecular mass of 324,910-Da. The encoded protein exhibits significant sequence similarity (47% identical residues and 65% conserved positions) to the previously characterized *fas*-like gene (16) of *B. ammoniagenes* (Fig. 2). In order to exclude strain-specific variations between the *fas* DNA analyzed in this work and that formerly studied by Meurer and coworkers (16), we reisolated distinct segments of both genes from a single *B. ammoniagenes* colony by PCR cloning. Sequencing of the isolated PCR fragments confirmed that both *fas* genes were indeed present in the same cell (data not shown). We therefore named the two genes *fasA* and *fasB*.

At the DNA level, *fasA* and *fasB* exhibit 61% overall sequence similarity. The similarity extends over the entire lengths of the genes, although to a variable extent. This colinearity suggests that the order of the catalytical FAS domains along the two multifunctional protein chains is identical (Fig. 2). Similarly, both FAS proteins correspond formally to a head-to-tail fusion of the yeast FAS subunits β and α, as was pointed out earlier for the *fasB* sequence (16). Unlike that of FasB, the amino acid sequence deduced for FasA was consistent with the experimentally determined N-terminal sequence of the purified *B. ammoniagenes* FAS (Fig. 2). From this it can be concluded that the *fasA* gene isolated in this study actually encodes the FAS predominantly produced in *B. ammoniagenes* cells. Since *fasA* and *fasB* are expected to encode proteins of very similar size, they ought to be copurified by the isolation procedure used. The predominance of FasA within the purified FAS preparation, as evidenced by N-terminal sequencing data, suggests that FasB is synthesized either at a very low level or only under specific conditions.

Differential expression rates of the two *B. ammoniagenes fas* genes. In order to assay the differential expression rates of *fasA* and *fasB*, specific antibodies were prepared against an internal 19-amino-acid peptide of FasB. This peptide, QVVSTDE GQAALFDVGQAP, corresponding to positions 336 to 355 in Fig. 2, exhibits very low sequence similarity to FasA. In contrast, the polyclonal antiserum prepared against purified *B. ammoniagenes* FasA presumably reacts with both FasA and FasB. Crude cell extracts of *B. ammoniagenes* wild-type and *fasB*-disrupted cells (see below) were subjected to Western blot analysis with the two FAS antisera mentioned. As demonstrated in Fig. 3, the antibodies against purified *B. ammoniagenes* FAS produced a strong immunopositive signal with both wild-type and *fasB*-disrupted cell extracts (Fig. 3A, lanes 1 and 2). On the other hand, the antibodies directed against the FasB peptide elicited only a faint, though distinct, immunopositive reaction with the wild-type extract (Fig. 3B, lane 2). As expected, this band was absent in *fasB*-disrupted cells (Fig. 3B, lane 1). These findings confirm that the FasB antiserum is indeed specific for this FAS variant. In addition, they demonstrate that *fasB* is not a silent gene but is expressed, under the conditions applied here, at a definite (though considerably lower) rate than *fasA*. Expression of both FAS variants is drastically increased in *B. ammoniagenes* transformants containing multiple copies of the respective *fasB* and *fasA* genes. The corresponding Western blot and SDS-PAGE patterns are shown in Fig. 3B and C, respectively. Essentially the same results were obtained with Northern (RNA) blot analyses with specific *fasA* and *fasB* hybridization probes. In these experiments, on the background of a considerable smear of degradation products, the *fasA* probe gave a strong signal while the *fasB* signal was not above background (data not shown). This suggests that the differential expression of the *fasA* and *fasB* genes may occur at the transcriptional level.

Disruption of *fasA* and *fasB* reading frames by insertional mutagenesis. In separate experiments, each of the two *B. ammoniagenes fas* genes was disrupted, at the chromosomal level, by targeted integration of either one of the suicide plasmids pDV2 and pECMΔSHM. These plasmids contained, respectively, the central 1.8-kb *Eco*RI fragment (BF1) of the *fasA* gene and a homologous 1.5-kb *Sph*I-*Hin*dIII fragment derived from the *fasB* gene. The suicide vector pECMΔ, which was used to construct both insertion plasmids, was derived from the *E. coli-B. ammoniagenes* shuttle vector pECM1 by elimination of the 2.4-kb *Sph*I fragment containing the DNA element responsible for autonomous replication in *B. ammoniagenes*. Transformants which had stably integrated the above-mentioned constructs were selected on fatty acid-containing media according to their acquired kanamycin resistance. The plasmid insertion was expected to destroy the *fas* reading frames by disrupting them into two halves (Fig. 4B). The proximal ends of both halves contain a duplicated *fas* sequence resulting from the recombinational event. By the same mechanism, the integrated DNA may be eliminated again, thereby restoring the original, functionally intact *fas* DNA. This occurs readily if the selective agent, kanamycin, is omitted from the growth media. For both the *fasA* and *fasB* genes, successful disruption was confirmed by Southern hybridization (Fig. 4A). In each case, the respective *fas* restriction fragments were shifted to the expected higher-molecular-mass positions in the gel.

The capacities of *fas*-inactivated *B. ammoniagenes* cells for saturated and unsaturated fatty acid biosynthesis were examined by replica plating onto fatty acid-free selective media and onto media supplemented with palmitic and oleic acid, respectively. It turned out that *fasB* disruptants exhibited no fatty acid requirement whatsoever. In contrast, *fasA* disruptants required oleic acid for growth and failed to grow on kanamycin-containing, fatty acid-free media. Palmitic or stearic acid supplementation did not allow growth under these conditions. These


B. ammoniagenes FAS-A
B. ammoniagenes FAS-B
AT
MPT
ACP

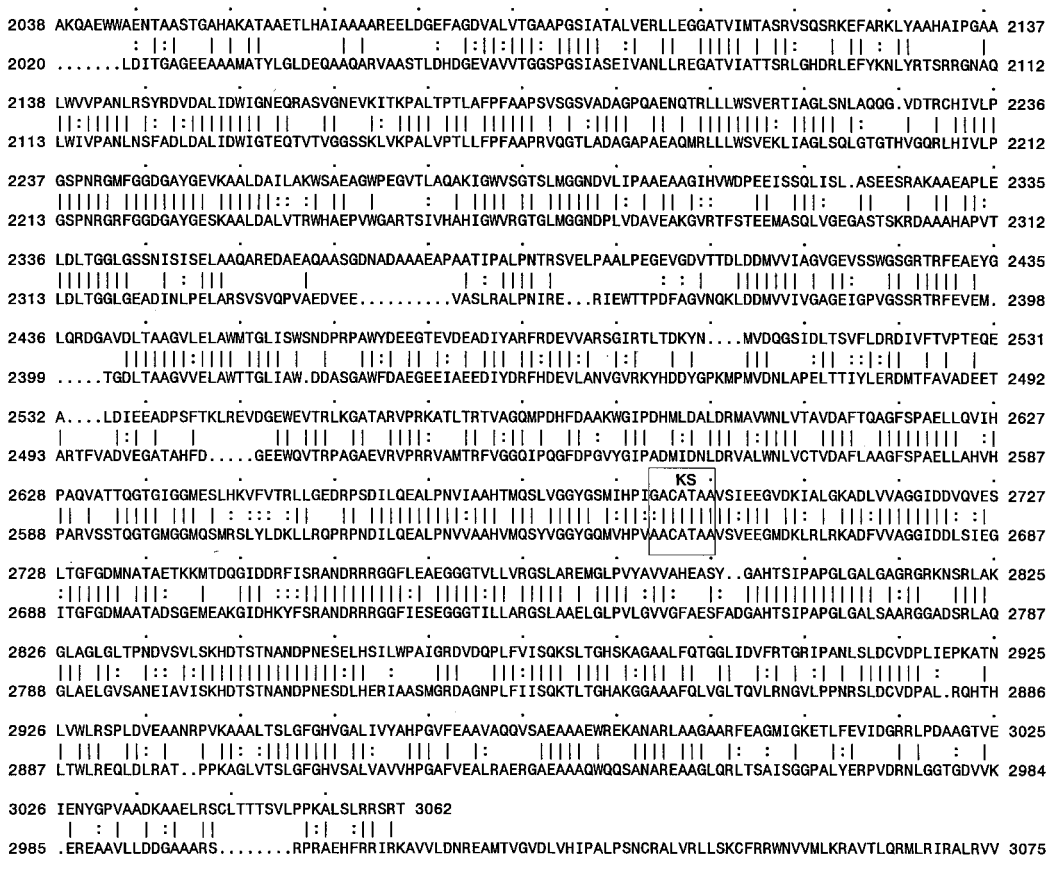

FIG. 2. Sequence comparison of FasA and FasB. The N-terminal amino acid sequence of purified *B. ammoniagenes* FAS and the consensus sequences of known substrate binding sites are boxed. The FasB sequence used for the preparation of a specific antiserum is underlined. Abbreviations: AT, acetyl transferase; MPT, malonyl-palmitoyl transferase; ACP, acyl carrier protein; KS, β-ketoacyl synthase. Identical amino acids (vertical lines) and conserved positions (double dots) are indicated.

findings suggest that the function of FasB is either dispensable or may be replaced by FasA, but not vice versa. Furthermore, saturated-fatty acid synthesis is obviously achieved by more than one FAS system in *B. ammoniagenes* cells.

Complementation of oleic acid-requiring *B. ammoniagenes* mutants by *fasA* DNA. Previous attempts in our laboratory to obtain, by ethyl methanesulfonate (EMS) mutagenesis, fatty acid-requiring *B. ammoniagenes* mutants resulted exclusively in the isolation of oleic acid-dependent strains (15). These results are consistent with the characteristics of the previously described *fasA* insertional mutants and confirm the idea that there exists only one oleic acid-synthesizing FAS system in *B. ammoniagenes* while the capacity to synthesize saturated fatty acids must be redundant. The differential biosynthetic characteristics of *fasA* and *fasB* were again demonstrated by transforming the EMS-induced oleic acid-requiring mutants alternatively with either one of the *fasA* or *fasB* DNA-containing plasmids. As is evident from Fig. 5, the wild-type phenotype was restored in *fasA*-transformed cells but not in *fasB*-transformed cells. These complementation data are consistent with the aforementioned *fas* inactivation results, confirming that *fasA* has the capacity to catalyze the synthesis of oleic acid but *fasB* does not.

The *B. ammoniagenes* transformations described in this work were performed by both conjugational plasmid transfer and electroporation. The efficiency of conjugational plasmid transfer to the *B. ammoniagenes* ATCC 6871 wild-type cells was in accordance with data described for the related strain *B. ammoniagenes* ATCC 6872 (20). In contrast, plasmid transfer to the oleic-acid-requiring mutants isolated in this work was significantly less efficient (<10% of that of the wild type). On the other hand, reproducibly high transformation rates for both wild-type and mutant cells were obtained by electroporation. However, with this technique, high transformation rates were observed only when plasmid amplification prior to transformation was carried out in *B. ammoniagenes* rather than in *E. coli* DH5α. Under optimal conditions, up to 10^5 transformants per μg of DNA could thus be obtained. The transformational inefficiency of the *E. coli*-derived plasmid DNA suggests the existence of a stringent restriction-modification system in *B. ammoniagenes* ATCC 6871. In this respect, *B. ammoniagenes* conforms to the characteristics of *Corynebacterium glutamicum* reported by Liebl et al. (10).

DISCUSSION

The isolation of *fasA*- and *fasB*-defective *B. ammoniagenes* mutants, as described in this study, provides the first insight into the differential functions of these two bacterial FAS complexes. If both enzymes were functionally identical, no phenotypically defective *fas* mutants should be observed. Indeed, insertional inactivation of *fasB* did not have any phenotypic

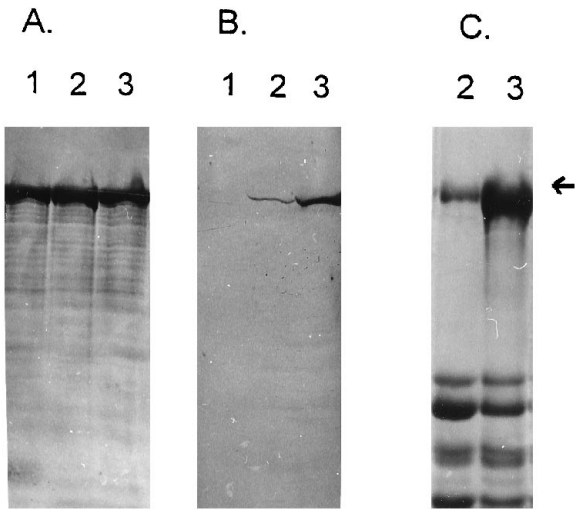

FIG. 3. Western blot (A and B) and SDS-PAGE (C) analyses of cell extracts from *B. ammoniagenes* wild-type (lanes 2), *fasB*-disrupted (lanes 1), and *fas*-transformed (lanes 3) cells. The antisera used were directed against purified *B. ammoniagenes* FAS (A) and against a specific FasB peptide (B). The polyclonal antiserum used in panel A was directed against purified *B. ammoniagenes* FAS and reacts with both the FasA and FasB proteins. The antiserum used in panel B was raised against a specific peptide and is inactive towards FasA. Transformation was performed with pHPS2 containing the *fasA* gene together with 3 kb of its 5′ flanking regions and 1 kb of its 3′ flanking regions (panel C, lane 3) or with pGH1 containing the *fasB* gene together with 0.5 kb of both its 5′ and 3′ flanking regions (B, lane 3). About 100 μg of cell protein was applied to each lane. Gels were either developed by immunostaining (A and B) or stained with Coomassie blue (C). The arrow indicates the position of the FAS proteins.

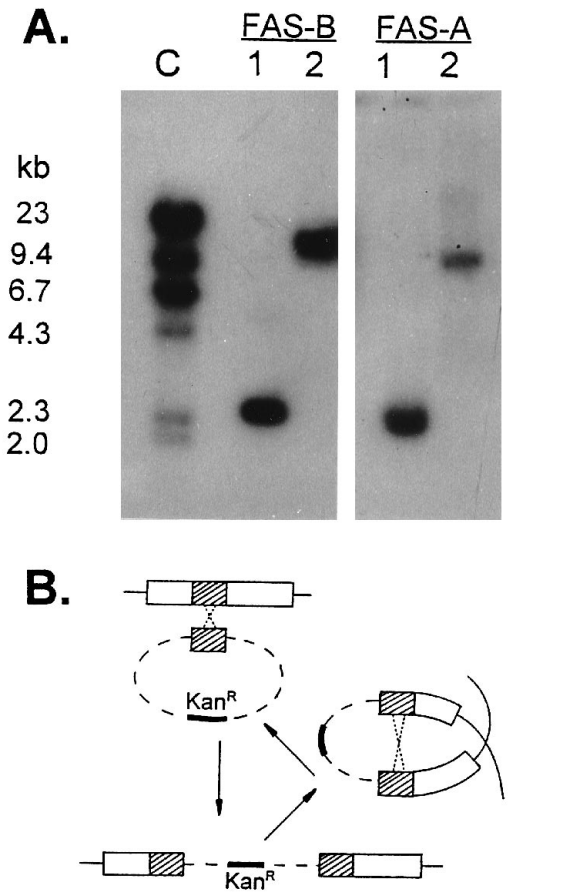

FIG. 4. Disruption of the *B. ammoniagenes fasA* and *fasB* genes. (A) Southern blot analysis of wild-type (lanes 1) and *fasB*- and *fasA*-disrupted (lanes 2) DNA after digestion with *Xho*I (*fasB*) and *Bst*EII (*fasA*), respectively. Hybridization was performed with the central *Sph*I-*Hin*dIII (*fasB*) and *Eco*RI (*fasA*) fragments, respectively. *Hin*dIII-digested lambda DNA was used as a size marker (C). (B) Scheme illustrating the recombinational integration and disintegration of the *fas*-containing plasmids into the chromosomal *fas* DNA (open bar). The internal *fas* region contained in the suicide vector is hatched. Kan^R, kanamycin resistance marker.

effect, suggesting that FasB is either redundant to FasA or serves a dispensable function under the conditions employed. On the other hand, the corresponding *fasA* mutants required oleate for growth. No palmitate-oleate double deficiencies were associated with any of the mutants, although this would be expected with the loss of an enzyme which synthesizes saturated and unsaturated fatty acids at the same time (5). Similarly, mutants exclusively requiring oleate were obtained in earlier experiments when a large-scale screening for EMS-induced *fas* mutants was performed (15). Obviously, the origin of saturated fatty acids in *fasA*-defective mutants lacking the predominant cellular FAS complex remains to be elucidated. As an alternative to the unaffected FasB enzyme, they could also originate from a third, as yet unidentified, possibly non-aggregated *B. ammoniagenes* FAS. Compared with these alternatives, it appears rather unlikely that any palmitate-synthesizing activity has remained with the two gene fragments produced by the *fasA* disruption process. The availability of the specifically *fasA*- or *fasB*-defective *B. ammoniagenes* mutants described in this study provides the opportunity to perform detailed in vitro biochemical studies on the biosynthetic capacities of both enzymes. From these mutants, homogeneous preparations of each Fas enzyme may now be isolated. In principle, only a mixture of both enzymes should be obtained from *B. ammoniagenes* wild-type cells. At least the minor variant, FasB, was thereby not accessible for biochemical characterization.

The three transacylase active sites as well as the reactive cysteinyl residue of the β-ketoacyl synthase have been assigned to distinct locations within FasA and FasB according to their sequence similarities to the corresponding sites within yeast FAS (Fig. 2). These yeast sites had originally been identified by substrate binding studies and by peptide sequencing of appropriate acyl enzyme intermediates (4, 8, 22). Similarly, the pantetheine-binding serine residue in yeast FAS was identified by sequencing of radioactively labelled holoenzyme fragments (22). So far, this analysis has not been performed with the two *B. ammoniagenes* type I FAS enzymes. Hence, the assignment of pantetheine binding sites in FasA and FasB indicated in Fig. 2 remains tentative and is based on their overall sequence similarities to the corresponding yeast FAS regions (16). In contrast to the aforementioned catalytic sites, the ketoreductase, enoyl reductase, and dehydratase domains cannot be labelled by substrate binding. Instead, they have been localized by deletion mapping of appropriate yeast FAS mutations (23). Because of the colinearity of their overall sequences, the respective domains in the *B. ammoniagenes fasA* and *fasB* genes have been attributed to positions corresponding to those of the yeast enzyme. In contrast to the activities involved in saturated fatty acid synthesis, nothing is known about the location of the components required for oleic acid production, i.e., the putative 3-hydroxydodecanoyl-FAS dehydratase and dodecenoyl-*CoA* Δ^3-*cis*-Δ^2-*trans*-isomerase. Comparison with the corresponding *E. coli* gene, *fabA* (3), revealed no obvious similarity to any part of the *B. ammoniagenes fasA* gene. Since FasA

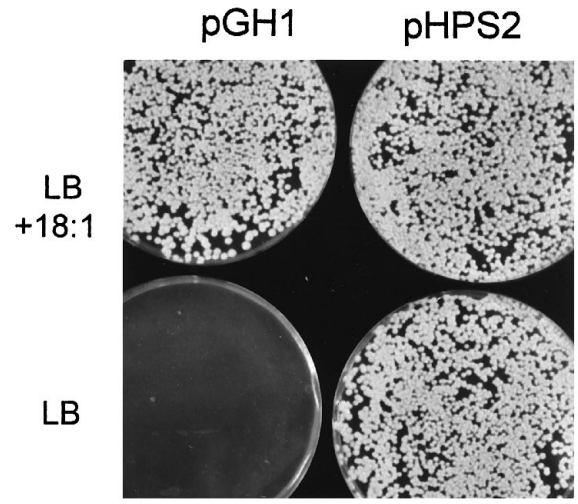

FIG. 5. Complementation of the oleic acid-requiring *B. ammoniagenes* mutant BA 15-04. The mutant cells were transformed by electroporation with the FasA expression plasmid pHPS2 and the FasB expression plasmid pGH1. Transformants were selected on kanamycin-containing Luria-Bertani plates with and without oleic acid supplementation.

catalyzes this reaction but FasB does not, the two FAS enzymes should characteristically differ, at least within restricted parts of their structures. A scrutinous comparison of FasA and FasB was performed to determine if this is the case. It revealed that apart from the colinearity and sequence similarity of FasA and FasB, in general there is specifically one region of about 200 amino acids in length where this similarity is rather low if significant at all (positions 860 to 1070 in Fig. 2). Therefore, future studies aimed at the characterization of enzymatic functions specifically involved in oleic acid biosynthesis may have to concentrate on this part of FasA.

ACKNOWLEDGMENT

This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie. In addition, financial support from the BIOTECH (B 102 CT-942067) and HCM (ERBC HRXCT 940570) programs of the European Commission is gratefully acknowledged.

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