Mutational Analysis of the *Rhizobium lupini* H13-3 and *Sinorhizobium meliloti* Flagellin Genes: Importance of Flagellin A for Flagellar Filament Structure and Transcriptional Regulation

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Complex flagellar filaments are unusual in their fine structure composed of flagellin dimers, in their right-handed helicity, and in their rigidity, which prevents a switch of handedness. The complex filaments of *Rhizobium lupini* H13-3 and those of *Sinorhizobium meliloti* are composed of three and four flagellin (Fla) subunits, respectively. The Fla-encoding genes, named *flaA* through *flaD*, are separately transcribed from σ^{28} -specific promoters. Mutational analysis of the *fla* genes revealed that, in both species, FlaA is the principal flagellin and that FlaB, FlaC, and FlaD are secondary. FlaA and at least one secondary Fla protein are required for assembling a functional flagellar filament. Western analysis revealed a ratio close to 1 of FlaA to the secondary Fla proteins (= FlaX) present in wild-type extracts, suggesting that the complex filament is assembled from FlaA-FlaX heterodimers. Whenever a given mutant combination of Fla prevented the assemblage of an intact filament, the biosynthesis of flagellin decreased dramatically. As shown in *S. meliloti* by reporter gene analysis, it is the transcription of *flaA*, but not of *flaB*, *flaC*, or *flaD*, that was down-regulated by such abortive combinations of Fla proteins. This autoregulation of *flaA* is unusual. We propose that any combination of Fla subunits incapable of assembling an intact filament jams the flagellar export channel and thus prevents the escape of an (as yet unidentified) anti- σ^{28} factor that antagonizes the σ^{28} -dependent transcription of *flaA*.

Motile bacteria are propelled by helical flagellar filaments connected by a proximal hook to the basal body holding the flagellar rotary motor (for reviews, see references 1, 20, and 41). Traditionally, flagellar filaments are classified by their electron microscopical appearance into two types, named plain and complex (22, 33). The plain filaments of *Escherichia coli* and *Salmonella* spp. have a smooth surface structure with faint striations, whereas the complex filaments of soil bacteria, like *Rhizobium lupini* H13-3 and *Sinorhizobium meliloti*, exhibit a prominent helical pattern of alternating ridges and grooves (16, 32, 38). Unlike the flexible plain filaments, which are capable of switching from left-handed to right-handed helicity (21), the complex filaments are more rigid and do not switch handedness. Pairwise helical perturbations result in a subunit composed of a dimer of flagellin (39). Interflagellin bonds are believed to lock the complex filament in a rigid, right-handed helical conformation suitable for propulsion in viscous media (4, 8). Concomitantly, the flagellar motor of *Rhizobium* rotates entirely clockwise and does not reverse its sense of rotation (9). It has been shown that swimming *S. meliloti* cells respond to tactic stimuli by modulating their flagellar rotary speed (37) and that two novel motor proteins may be essential players in speed control (27). Hence, directional changes in the tracks of swimming *S. meliloti* cells—imperative for any chemotactic response—are a consequence of individual flagella rotating at different speeds (31). It thus appears that complex flagellar filaments and the new mode of directional control of swimming

cells have evolved in response to the specific condition of swimming in viscous fluids prevailing in the soil biotope.

The flagellar filament consists of an assembly of about 20,000 flagellin subunits, whose molecular mass typically ranges from 40 to 60 kDa (20). Flagellins are three-domain proteins, with the N- and C-terminal domains being responsible for the quaternary interactions between subunits and the central, surface-exposed domain performing no obvious structural role but containing all of the potent antigenic epitopes. We have previously shown that the *S. meliloti* genome contains four genes, *flaA*, *flaB*, *flaC*, and *flaD*, encoding four related flagellin subunits (28, 36), and we report here three flagellin genes, *flaA*, *flaB*, and *flaD*, as constituents of the *R. lupini* H13-3 flagellar regulon. The latter strain was chosen because the first 13-Å-resolution, three-dimensional density map has been generated from its complex filament using low-dose electron micrographs of negatively stained specimens (4). This constellation may provide specific handles for future sequence-structure analysis.

In an effort to understand the process of assembling the complex filaments of the related soil bacteria *R. lupini* H13-3 and *S. meliloti* and to elucidate the contribution of single subunits to the filament structure, we have taken a genetic approach. Mutational analyses revealed that in both strains flagellin A is the principal, absolutely essential subunit but that, in addition, at least one of the secondary flagellin species is needed for assembling a functional filament. We also report that flagellin A biosynthesis is subject to control by transcriptional regulation.

MATERIALS AND METHODS

Bacteria and plasmids. Derivatives of *E. coli* K-12, *R. lupini* H13-3 (7), and *S. meliloti* MV II-1 (15) and the plasmids used are listed in Table 1.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Markers ^a and derivation	Source or reference(s)
Strains		
<i>E. coli</i>		
DH10B	<i>recA1 endA1</i>	GIBCO/BRL
S17-1	<i>recA endA thi hsdR</i> RP4-2 Tc::Mu::Tn7 T ^r Sm ^r	34
<i>S. meliloti</i>		
RU11/001	Sm ^r spontaneous streptomycin-resistant wild-type strain	29
RU11/011	Sm ^r Nm ^r Δ <i>flaA</i> Δ <i>flaB</i> Δ <i>flaC</i> Δ <i>flaD</i>	29, 35
RU11/820	Sm ^r Δ <i>flaA</i>	This work
RU11/821	Sm ^r Δ <i>flaB</i>	This work
RU11/822	Sm ^r Δ <i>flaC</i>	This work
RU11/823	Sm ^r Δ <i>flaD</i>	This work
RU11/824	Sm ^r Δ <i>flaB</i> Δ <i>flaC</i>	This work
RU11/825	Sm ^r Δ <i>flaB</i> Δ <i>flaD</i>	This work
RU11/826	Sm ^r Δ <i>flaC</i> Δ <i>flaD</i>	This work
RU11/833	Sm ^r Δ <i>flaA</i> Δ <i>flaB</i>	This work
RU11/834	Sm ^r Δ <i>flaA</i> Δ <i>flaC</i>	This work
RU11/835	Sm ^r Δ <i>flaA</i> Δ <i>flaB</i> Δ <i>flaD</i>	This work
RU11/836	Sm ^r Δ <i>flaA</i> Δ <i>flaC</i> Δ <i>flaD</i>	This work
RU11/837	Sm ^r Δ <i>flaB</i> Δ <i>flaC</i> Δ <i>flaD</i>	This work
RU11/842	Sm ^r Δ <i>flaA</i> Δ <i>flaB</i> Δ <i>flaC</i>	This work
<i>R. lupini</i>		
RU12/001	Sm ^r , spontaneous streptomycin-resistant wild-type strain	This work
RU12/002	Sm ^r Δ <i>flaA</i>	This work
RU12/003	Sm ^r Δ <i>flaB</i>	This work
RU12/004	Sm ^r Δ <i>flaD</i>	This work
RU12/005	Sm ^r Δ <i>flaB</i> Δ <i>flaD</i>	This work
RU12/006	Sm ^r Δ <i>flaA</i> Δ <i>flaB</i> Δ <i>flaD</i>	This work
RU12/007	Sm ^r Δ <i>flaA</i> Δ <i>flaD</i>	This work
Plasmids		
pK18 <i>mobsacB</i>	Km ^r ^b <i>lacZ</i> <i>mob</i> <i>sacB</i>	30
pPHU234 ^c	Tc ^r , promoterless <i>lacZ</i> fusion	14
pUCBM20	Ap ^r	Boehringer Mannheim
pRU738	Ap ^r Tc ^r (<i>tet</i>)	40
pRU1993	Km ^r ^b Tc ^r , recombinant of a 3,028-bp <i>EcoRI</i> / <i>PstI</i> <i>tet</i> gene from pRU738 and pK18 <i>mobsacB</i>	25
pRU2274	Tc ^r , recombinant of a 545-bp <i>EcoRI</i> / <i>PstI</i> fragment containing the <i>flaA</i> promoter of <i>S. meliloti</i> and pPHU236	35
pRU2275	Tc ^r , recombinant of a 263-bp <i>flaB</i> <i>EcoRI</i> / <i>PstI</i> fragment of the <i>flaB</i> promoter of <i>S. meliloti</i> and pPHU236	25
pRU2276	Tc ^r , recombinant of a 397-bp <i>flaD</i> <i>XhoI</i> / <i>PstI</i> fragment of the <i>flaD</i> promoter of <i>S. meliloti</i> and pPHU234	25
pRU2277	Tc ^r , recombinant of a 311-bp <i>flaC</i> <i>EcoRI</i> / <i>PstI</i> fragment of the <i>flaC</i> promoter of <i>S. meliloti</i> and pPHU235	25
pRU2351	Km ^r Tc ^r , recombinant of a 10.7-kb genomic <i>MluI</i> fragment containing <i>flaA</i> and <i>flaB</i> of <i>R. lupini</i> RU12/001 and pRU1993	This work
pRU2352	Km ^r Tc ^r , recombinant of a 8.9-kb genomic <i>HindIII</i> / <i>PstI</i> fragment containing <i>flaD</i> of <i>R. lupini</i> RU12/001 and pRU1993	This work

^a Nomenclature is according to the work of Bachman (2) and Novick et al. (26).

^b Selection for the Km^r marker in *S. meliloti* and *R. lupini* was on neomycin (120 mg/liter).

^c pPHU235 and pPHU236 are derivatives of pPHU234 with a different translation phasing of *lacZ*.

Media and growth conditions. *E. coli* strains were grown in Luria broth (19) at 37°C. *R. lupini* and *S. meliloti* strains were grown in TYC (0.5% tryptone, 0.3% yeast extract, 0.13% CaCl₂ · 6H₂O [pH 7.0]) at 30°C (27). Motile cells prepared for functional tests were grown for 2 days in TYC, diluted in 15 ml of RB minimal medium (8) to an optical density at 600 nm (OD₆₀₀) of 0.05, layered on Bromfield agar plates (37), and incubated on a slowly rotating platform at 30°C for 16 h to an OD₆₀₀ of 0.2 to 0.5. The following antibiotics were used at the indicated final concentrations: for *E. coli*, ampicillin at 100 mg/liter, kanamycin at 50 mg/liter, and tetracycline at 10 mg/liter; for *R. lupini* and *S. meliloti*, neomycin at 120 mg/liter, streptomycin at 600 mg/liter and tetracycline at 10 mg/liter.

Gene replacement and reporter gene assay. Deletions (listed in Table 1) were generated in vitro by overlap extension PCR as described by Higuchi (12). Constructs containing the deletions were cloned into the mobilizable suicide vector pK18*mobsacB*, used to transform *E. coli* S17-1, and then conjugally transferred to *R. lupini* or *S. meliloti* by filter matings according to the method of Simon et al. (34). Allelic replacement was achieved by sequential selections on neomycin and 10% sucrose as described previously (37). Confirmation of allelic replacement and elimination of the vector was obtained by gene-specific primer PCR and Southern blotting. The broad-host-range plasmid pPHU234 and its derivatives pPHU235 and pPHU236 served as vectors for translational fusions of

the four *S. meliloti* *fla* promoters. The resulting *lacZ* fusion plasmids were used to transform *E. coli* S17-1 and were then conjugally transferred to *R. lupini* RU12/001 or *S. meliloti* RU11/001 by streptomycin-tetracycline double selection, as described by Labes et al. (17).

DNA methods. *R. lupini* and *S. meliloti* DNAs were isolated and purified as described previously (37). Plasmid DNA was purified with NucleoSpin (Macherey Nagel, Düren, Germany). DNA fragments or PCR products were purified from agarose gels by use of a QiaEx DNA purification kit (Qiagen, Hilden, Germany). PCR amplification of chromosomal DNA and Southern blotting were carried out according to published protocols (27, 36). *R. lupini* genomic fragments containing the *fla* genes (Fig. 1) were isolated by plasmid insertion and rescue. An 850-bp *flaA* fragment and a 300-bp *flaD* fragment were PCR amplified and ligated into pRU1993, a derivative of the broad-host-range mobilizable vector pK18*mobsacB* containing the tetracycline resistance cassette of 2pRU738 (40). Recombinant plasmids were conjugally transferred and allowed to insert into the homologous *R. lupini* gene loci by a single crossover. Cells containing the insertion were selected on TYC plates containing neomycin and streptomycin. Chromosomal DNA was digested with *MluI* (*flaA*) or *HindIII* (*flaD*), ligated, and used to transform *E. coli* DH10B. Plasmid DNAs isolated from tetracycline-resistant transformants were physically mapped and sequenced with a model 310

FIG. 1. Aligned partial maps of the *S. meliloti* (*S.m.*) (36), *R. lupini* (*R.l.*) (this work) and *A. tumefaciens* (*A.t.*) (5) flagellar gene clusters. Solid lines signify fully sequenced genomic regions, and dashed lines signify partially sequenced genomic regions. Gene loci are not drawn to scale; relevant transcription units and their polarities are marked by small dashed arrows, and general transcription polarity is marked by large arrows. The nomenclature of the *S. meliloti* *flaC* and *flaD* genes (36) has been changed in accordance with their map order, and the *R. lupini* *flaD* gene has been named by analogy to its *A. tumefaciens* paralogue (5). An *R. lupini* *flaC* paralogue was not detected. *R. lupini* (and *A. tumefaciens*) genes translocated and inverted with respect to their positions on the *S. meliloti* map are connected by dotted lines.

automatic sequencer (Applied Biosystems, Weiterstadt, Germany). Sequences were aligned and compared by using Genetics Computer Group sequence analysis software (6).

Chemotaxis assays. Swarm plates containing Bromfield medium and 0.3% Bacto Agar were inoculated with 3- μ l droplets of the test culture and incubated at 30°C for 3 days. The speed of swimming cells was measured by using the computerized motion analysis of the Hobson BacTracker system (Hobson Tracking Systems Ltd., Leicester, United Kingdom) as described by Sourjik and Schmitt (37).

Flagellar filament isolation. Flagella were detached from motile cells (from 200-ml cultures) by agitation in a Braun MX32 mixer (Braun, Melsungen, Germany) at maximum power for 20 s, separated from cells by centrifugation at 8,000 $\times$ g, and purified by serial centrifugation at 8,000 $\times$ g for 8 min and at 15,000 $\times$ g for 15 min. Purified flagella sedimented at 87,000 $\times$ g for 2 h were washed once with a solution containing 0.5 mM CaCl₂, 0.1 mM EDTA, and 20 mM HEPES (pH 7.2) and resuspended in 150 μ l of the buffer. Ninety-five percent of the final preparation was flagella, as assessed by electrophoresis in 10% acrylamide gels in the presence of sodium dodecyl sulfate.

Immunoblots. Polyclonal antibodies raised against purified *R. lupini* and *S. meliloti* whole flagellar filaments were isolated from whole serum by affinity purification. One milligram of isolated flagellar filaments was resuspended in 450 μ l of sodium dodecyl sulfate sample buffer and heated to 100°C for 8 min. Fla proteins were separated electrophoretically in a 10% acrylamide gel by the method of Laemmli (18) and transferred to nitrocellulose (35). Blotted flagellins were cut into four pieces (5 by 20 mm²) to fit a 2-ml Eppendorf cup. Two milliliters of undiluted antiserum was added and incubated for 16 h at 4°C. Filter chips were washed three times with phosphate-buffered saline (PBS)-0.1% bovine serum albumin (BSA), twice with PBS-0.1% BSA-0.1% Nonidet P-40, and three times with PBS-0.1% BSA (5 min each). Bound antibodies were eluted from filter chips by mixing them carefully with 750 μ l of 0.2 M glycine-HCl, pH 2.5, for 1 min. The supernatant was immediately added to 375 μ l of prechilled KP_i (pH 9.0). The elution procedure was repeated twice, and the combined eluates were dialyzed three times against PBS. Whole-cell extracts were separated in a 10% acrylamide gel, transferred to nitrocellulose (as before), and probed as described previously (35) using purified anti-*R. lupini* or anti-*S. meliloti* Fla polyclonal antibody at a 1:500 dilution.

Protein and β -galactosidase assays. Protein concentrations were determined by the Bradford microassay (Bio-Rad, Munich, Germany). Cultures of *S. meliloti* containing *lacZ* fusions were sampled, diluted in Z buffer (24) to an OD₆₀₀ of 0.2,

permeabilized with 1 drop of toluene, and assayed for β -galactosidase activity by the method of Miller (24).

Electron microscopy. Complex flagella were negatively stained with uranyl acetate (3%, wt/vol) on carbon-coated copper grids. Images were taken on a transmission electron microscope (Philips model CM12) at 120 kV with a single-stage charge-coupled-device camera (Tietz, Gauting, Germany).

Nucleotide sequence accession numbers. The *R. lupini* *fla* sequences have been deposited in the GenBank database under accession no. AY7305, AY7306, and AY7307, and the *S. meliloti* *fla* sequences have been deposited under accession no. L49337.

RESULTS

Flagellin genes and deduced polypeptide sequences. Similarities between the *fla* genes of *R. lupini* H13-3 and *S. meliloti* (28, 36) were used in the design of *flaA* and *flaD* sequence-specific PCR primers (containing suitable restriction sites) that were used to amplify two gene-specific *R. lupini* DNA fragments. These were used as probes for plasmid rescue of two genomic, 10.7- and 8.7-kb regions containing *flaA-flaB* (pRU2351) and *flaD* (pRU2352), respectively. The *R. lupini* *fla* genes were completely sequenced and identified by the similarity of the encoded proteins to other flagellins, in particular those of *S. meliloti* (28, 36) and *Agrobacterium tumefaciens* (3, 5). Adjacent portions of the two clones were sequenced only to the extent where flagellar and motility genes could be identified and arranged on the map by similarity to their *S. meliloti* paralogues (36).

As shown in Fig. 1, the *R. lupini* *flaA* and *flaB* genes are oriented in a tandem array but separately from the third gene, *flaD*, located inversely on a distant portion of the flagellar regulon. This gene organization resembles that of *A. tumefaciens* (5), a close relative within the α subgroup of proteobacteria (10). Plausibly, this gene order is a consequence of trans-

R.1. <i>FlaA</i>	MASVLTNINAMSAALQTLRSISSNMEDTQSRISSGMRVGSASDNAAWWSIATTMRSNDASLSAVQDAIGLGAAKVD	
R.1. <i>FlaB</i>	-T-I--AA--A--T-GQ--N--ASV--L--A--GA--L--	
R.1. <i>FlaD</i>	-T-I--AA--A--M-DK-L-T--A-V--Y--ET-A--S--A--L--	
S.m. <i>FlaA</i>	-T-I--NS--A--SGV--S--L--QA--L--	
S.m. <i>FlaB</i>	-T-I--TA--AV--T-G--E--AHV--L--Q-A--MA--L--V--	
S.m. <i>FlaC</i>	-T-I--VA--A--G-D--E--A-V--L--T--V--MA--L--	
S.m. <i>FlaD</i>	-T-IM--PA--A--A-NH-L-T--G--F--ET-A--A--T-H--L--	
Consensus	MTSILTN AAMAALQTLR I SNME TQARISSGLRVG ASDNAAWWSIATTMRSND ALSAVQDALGLGAAKVD	
R.1. <i>FlaA</i>	TASAGMDAVIDVVKQIKNKLVTQAESSADKTKIQGEVKQLQEQLKGIVDSASFSGENWLKGDLS	T.....TT
R.1. <i>FlaB</i>	-----ESI-----A-T-DGV-KA-----D-I--I-----	
R.1. <i>FlaD</i>	--YDALANS-E--K--E--A-YGVG--RG--D-I--STSE-----QASI--NGGTPPVVEPI	
S.m. <i>FlaA</i>	--YS--ESA-E--E--A--A-T-DGV--A--E-IT--KD--TS-A-A-----QA--GG.....AV	
S.m. <i>FlaB</i>	--YS--ESA-E--E--K--A-T-DGV--A--E-ID--KD--TS-SEA-----QA--GG.....AV	
S.m. <i>FlaC</i>	--Y--ENAVE--E-RA--A-T-DGV--A--E-IE--KQ--TS-ATA-----QA--T.A.....PV	
S.m. <i>FlaD</i>	-FYSA-NT--MTE--A--A-S-PGV--D--NK--AE-KS--NSAAQ-----YNGAAALG.....	
Consensus	TAY GME IEVVKEIK KLVAATEDGVDKAKIQ EI QLK QL SI SASFSGENWLQADLS	
R.1. <i>FlaA</i>	TKSVVGSFVREG.GT VSVKTI DYALNASKVLVDTRATGKTGILDT..AYTGLNANTVTVDINKGGVITO..ASV	
R.1. <i>FlaB</i>	--T-----G-T-D-----	
R.1. <i>FlaD</i>	--K--A--T-T-S-N-G-T-V--V-DG-A--F--LS-G-L--KSAVFVAKTEQQI--QTSTTA--T-NAGYV--	
S.m. <i>FlaA</i>	-----D-S-S-A--KV--S--NS--F--V--D--K--V--NVSQAS--LTV--TN--ES--HT--	
S.m. <i>FlaB</i>	-----DAS-A--KV--S--TNS--F--V--N--K--V--NVSQAS--LT--TN--AS--HT--	
S.m. <i>FlaC</i>	-----DSS-V--V--DGNS--F--V--NN--K--V--DVSESS--LS--T--VSE--HT--	
S.m. <i>FlaD</i>	---I-A--N-SAD-S-T-S-LN-D,T-KS--I--V-DPSR-ML.....KAVDADALQSTPTGTA	
Consensus	TK SVVGSFVR GSG VSVKT DY L A SVLFDT. G TGILDK..VY. VT INT GV SQ..TV	
R.1. <i>FlaA</i>	RAYSTDEMLSLGAKVDGANSNVAV..GGGSAFVKV.DGSWVKGSVDAAASITASTPVAGKFAAA...YTAAEAG	
R.1. <i>FlaB</i>	-----A--N--Q--V-----	
R.1. <i>FlaD</i>	KKLTDA-IGT-N-ATPDT-ADPS-YSN-TVNYLRLSENT--VTATNPS-G-TTVAA-YRDTGSNDWF-DTTG-P	
S.m. <i>FlaA</i>	A--LESLTEA--EFQ--Y-L..Q--NSY--ENV--RAE.....A-GAT-QEI--T-T--	
S.m. <i>FlaB</i>	A--LESLTEA--EFQ--Y-L..Q--NSY--NV--RAE.....A-GAT-QEL--T-T--	
S.m. <i>FlaC</i>	A--TV-DLIAG--VFQ--Y-L..A--VNY--E-V--EA.....V--SGAP-QEV--V-T--A	
S.m. <i>FlaD</i>	-N-Y...LIDA--AP--ATEIENDN.....T-GA.....	
Consensus	AYS L GA G...N A ..GG YVKV. WVTA TGA G AA....TTA.AG	
R.1. <i>FlaA</i>	TAAAGDAIIVDETN.SGAGAVNLTQSVLTMVDSM.SSTDVGSYLTVGEKALTSLSAGAELSGIKQRIDLQVD	
R.1. <i>FlaB</i>	---V---N---T---TL---K---A---M---E---	
R.1. <i>FlaD</i>	-SV-RSLGLS-STLDLDNLVD-AAAM-GFSGGTNYTA-DAIDVMMSE-D-Q-EAM--TASS--LQS--NM-EN	
S.m. <i>FlaA</i>	-IT--SWV--VG-.AP-AN-SAG--ANINIVG-G.AAALDALIS--DA--DM--A-S--SS--SE	
S.m. <i>FlaB</i>	-IT--SWV--VG-.AP-AN-SAG--AGINIVG-G.AVRLDALIS--DA--DM--A-D--AM--S-	
S.m. <i>FlaC</i>	PIT--SWA--T-A.GP-AS-PAPA-IENI-ITNAAQAANLDALIR--DE--ED-I--TSA--SM--GM-EE	
S.m. <i>FlaD</i>	QLGDMISV-DELISQ--DSA-T--A-TS--EM-ES	
Consensus	T A..D VD N. V A QSV M LD ISGVD ALT LTSAAA LGS I RIDLQE	
R.1. <i>FlaA</i>	FASKIGDALAKGIGRLVDADMNEESTKLKALQTQQQLAIQSLSIANSDSQNILSLFR	410 -- --
R.1. <i>FlaB</i>	---S-SID-----TS-E-----Q	411 87/90
R.1. <i>FlaD</i>	-V-T-M-VID-----R-----G--A--ANA--Q--K	430 49/55
S.m. <i>FlaA</i>	-VN--S-SIES-V-----R-----A-----V-----	395 61/68
S.m. <i>FlaB</i>	-VN--S-SIDS-V-----R-----A-E-V-T--	395 59/66
S.m. <i>FlaC</i>	-V--T-SIDS-----R-----TN-E--Q--Q	395 58/65
S.m. <i>FlaD</i>	-VAN-M-VID--V-----R-----G-----TT-E--RL-QE	321 56/63
Consensus	FVSKL DSIDKGIGRLVDADMNEESTRLKALQTQQQLAIQSLSIAN SENIL LFR	

FIG. 2. Comparison of the three *R. lupini* (R.1.) and four *S. meliloti* (S.m.) deduced flagellin polypeptide sequences. Numbering refers to amino acid residues in each line. Dashes signify identical residues with respect to the *R. lupini* FlaA sequence, and dots signify gaps. The consensus sequence includes all residues that form a homology group with a weighted relative frequency of 0.5 or greater. Conserved N- and C-terminal domains are boxed, and black bars denote amino acid residues unique to right-handed helical flagellar filaments (41). Identity/similarity values (percentages) relative to *R. lupini* FlaA are listed at the end of each sequence.

location and inversion of the flagellar regulon downstream of *flaC* on the *S. meliloti* map, as delineated in Fig. 1. This event may have eliminated the *flaC* paralogue from the *R. lupini*, but not from the *A. tumefaciens* genome (see also below). The *R. lupini* *flaD* gene has been named in accordance with its *A. tumefaciens* paralogue, because of their congruent map locations and the high similarity (87%) of the encoded flagellins.

The three *R. lupini* flagellin genes, *flaA* (1,230 bp), *flaB*

(1,233 bp), and *flaD* (1,290 bp), encode polypeptides of 410 (41.9 kDa), 411 (42.1 kDa), and 430 (45.2 kDa) amino acid residues, respectively. These show significant similarity to the four flagellin subunits of the *S. meliloti* complex filaments, as revealed by sequence alignments shown in Fig. 2. The amino- and carboxy-terminal regions (Fig. 2) thought to determine the intra- and intermolecular interactions that define the basic filament structure (13) are highly conserved. Notably, four se-

FIG. 3. Dendrogram based on full-length flagellin sequences from *A. tumefaciens* (Fla A. t.), *R. lupini* (Fla R. l.), and *S. meliloti* (Fla S. m.) and constructed by using the progressive, pairwise alignment method of PileUp from the Genetics Computer Group package (6). The *Salmonella enterica* serovar Typhimurium flagellin (FlaC S. t.) sequence was used as an outgroup marker.

quence motifs within these terminal regions (Fig. 2) are unique to filaments that form right-handed helices (41). The central domain defining the antigenically and structurally diverse filament surface exhibits some variability among flagellins of *R. lupini* and *S. meliloti* complex filaments but differs strongly from those of *A. tumefaciens* flagellins (not shown). In accord with sequence variations in the exposed central domain, only weak cross-reactions (<5%) of anti-*R. lupini* Fla antibody with *S. meliloti* flagellin and vice versa have been observed. Close similarities between the various flagellins of the three α subgroups of proteobacteria were also revealed by a phylogenetic analysis (Fig. 3). It appears that, for each species, FlaD is ancestral and that the other subunits are paralogues generated by gene duplication and diversion. Complex filaments, in addition to distinctive features of their domain compositions, are outstanding by their dimeric subunit structure (4, 38, 39). In view of the dominance of FlaA in assembling a functional filament (see below), it is curious that the secondary flagellins (FlaB, FlaC, and FlaD) of neither *R. lupini* nor *S. meliloti* differ significantly from the cognant FlaA sequence (Fig. 2). Subtle but consistent differences between the conserved termini of FlaA and the other Fla polypeptides are promising candidates for further investigating this aspect.

Mutational analysis of flagellin genes. To study the contribution of single flagellin subunits to flagellar filament function and structure, the three *fla* genes of *R. lupini* and the four *fla* genes of *S. meliloti* were separately inactivated by introducing

defined deletions using allelic replacement (30). Although our previous results (28) and gene mapping (36) (Fig. 1) indicated that each *fla* gene is transcribed from its own promoter, in-frame deletions were generated throughout to minimize the chance of polar effects. Single- and multiple-allelic *fla* mutants were tested for their motile behavior and for flagellar morphology. Motile fitness was determined on swarm plates (by comparison to that of the wild type) and by computerized motion analysis of free-swimming cells (37). The averaged data of all *R. lupini* and *S. meliloti* strains tested are listed in Table 2. The table also lists features of flagellar appearance, as inspected by electron microscopy (Fig. 4). The wild-type specimen (Fig. 4A and D) exhibited the typical cross-hatched pattern reflecting the conspicuous helical undulations of complex flagellar filaments (32, 33). The two FlaA-deficient filaments shown failed to form proper helical filaments and were quite fragile (Fig. 4B and E), although their surface fine structure appeared to be normal (Fig. 4C and F).

The overall results of functional and structural analyses listed in Table 2 were consistent for the two species and may be summarized as follows. (i) FlaA is the principal flagellin and is required for assemblage of a functional filament. Although *flaA* deletion mutants still produced short filaments (consisting of the secondary flagellin subunits FlaB and FlaD [and FlaC in *S. meliloti*]) with apparently normal fine structures (Fig. 4C and F), these filaments were straight or exhibited enhanced curvature, appeared unusually fragile, and were nonfunctional. (ii) FlaA is essential but not sufficient for the assemblage of functional filaments. In mutants carrying deletions of all secondary *fla* genes, FlaA alone produced merely a few severely truncated, frequently unstructured (*S. meliloti*) surface extrusions that were nonfunctional (not shown). (iii) Any combination of FlaA and one secondary flagellin is functional, although such a combination produces reduced swarming and swimming efficiency, compared to those aspects of the wild type (Table 2). Swimming efficiency obviously depends on the number and amounts of accessory flagellins present, and it was only the complete set of accessory flagellins that, together with FlaA, resulted in a sufficient number of fully functional flagellar filaments per cell. It thus appears that the complex filaments of *R. lupini* and *S. meliloti* are assembled from heterodimeric FlaA-FlaX subunits (where FlaX symbolizes one or several accessory Fla proteins). The question of whether this requires a 1:1 stoichiometry of FlaA-FlaX or not was approached by measuring the expression of individual *fla* genes.

Western blot analysis. Approximate amounts of flagellin produced by motile wild-type and *fla* mutant cells of *R. lupini* and *S. meliloti* were compared by Western blot analysis. Flagellin bands were detected by purified polyclonal anti-flagellin antibody, as shown in Fig. 5. Wild-type *R. lupini* (Fig. 5A) presented two bands of 41 kDa (FlaA) and 42 kDa (FlaB). A third band, corresponding to FlaD, was not detectable, probably owing to low gene expression, and there was no indication whatsoever of a fourth flagellin subunit. Most striking was the decrease in flagellin in two mutants ($\Delta flaAD$ and $\Delta flaBD$) (Fig. 5A) that could not assemble a functional filament, because either the principal FlaA subunit or the accessory FlaB and FlaD subunits were missing. This observation was augmented and confirmed by the immunochemical analysis of *S. meliloti* (Fig. 5B). In this species, FlaA and FlaB comigrated at 43 kDa,

TABLE 2. Properties of *R. lupini* and *S. meliloti* flagellin mutants

Organism	Relevant genotype	Effective Fla subunit(s)	Swarm size (%) ^a	V_{inst} ($\mu\text{m/s}$) ^b	Filament morphology ^c
<i>R. lupini</i>	Wild type	ABD	100	37.3 ± 3.4	Normal
	$\Delta flaA$	BD	0	0	Straight, severely truncated (Fig. 4B)
	$\Delta flaB$	AD	93 ± 3	36.1 ± 3.2	Normal
	$\Delta flaD$	AB	74 ± 6	35.1 ± 2.9	Normal
	$\Delta flaAD$	B	0	0	Straight, severely truncated
	$\Delta flaBD$	A	0	0	Straight, (numbers low)
	$\Delta flaABD$		0	0	No flagella
<i>S. meliloti</i>	Wild type	ABCD	100	38.6 ± 3.1	Normal
	$\Delta flaA$	BCD	6 ± 2	— ^e	Enhanced curvature, severely truncated (Fig. 4E)
	$\Delta flaB$	ACD	19 ± 1	26.6 ± 2.4	Normal (reduced numbers)
	$\Delta flaC$	ABD	39 ± 4	30.7 ± 2.0	Normal (reduced numbers)
	$\Delta flaD$	ABC	62 ± 2	36.2 ± 3.3	Normal, fragile
	$\Delta flaAB$	CD	0	0	ND ^d
	$\Delta flaAC$	BD	0	0	ND
	$\Delta flaBC$	AD	7 ± 3	20.5 ± 1.8	Normal (reduced numbers)
	$\Delta flaBD$	AC	21 ± 2	25.7 ± 1.6	ND
	$\Delta flaCD$	AB	26 ± 2	26.3 ± 2.1	ND
	$\Delta flaABD$	C	0	0	No flagella
	$\Delta flaACD$	B	0	0	No flagella
	$\Delta flaBCD$	A	0	0	Rare extensions, no fine structure
	$\Delta flaABCD$		0	0	No flagella

^a Percentage of wild-type swarm diameter (after deduction of 6-mm diameter of inoculum) on 0.3% Bromfield agar after 36 h at 30°C. Values are means of results from four replicates.

^b Instantaneous velocity (absolute speed) averaged for every swimming track. Mean values for 1,000 individual tracks were determined from each sample by computerized motion analysis and were averaged from at least five independent cell populations.

^c Appearance on electron micrographs.

^d ND, not determined.

^e —, the movement was jiggly.

FlaC appeared as a weak, slightly slower-migrating band (Fig. 5B) ($\Delta flaAB$ and $\Delta flaBD$), and FlaD was not detectable. Like in *R. lupini*, the amounts of Fla proteins synthesized decreased dramatically if the given subunit combination prevented the assemblage of a functional filament, notably, if FlaA and one or several secondary Fla subunits were lacking. Therefore, there must be a feedback regulation that reduces the rate of flagellin biosynthesis whenever an unproductive combination of Fla subunits prevents the assemblage of functional flagellar filaments. An apparent exception to this rule is the lack of reduction in (secondary) flagellin synthesis seen in $\Delta flaA$ mutants of both *R. lupini* and *S. meliloti* (Fig. 5A and B). This lack of reduction is considered a consequence of transcriptional down-regulation that affects only *flaA* and not the other *fla* genes, as shown below (Table 3).

Transcriptional control of *fla* genes. When the general picture of a feedback control of flagellin biosynthesis by nonfunctional subunit combinations emerged, we asked whether this regulation relates to *fla* gene transcription or not. Transcription from the four *S. meliloti* *fla* gene promoters was probed by using *lacZ* fusions in wild-type and *fla* mutant backgrounds. Table 3 lists the β -galactosidase activities expressed as fractions of the wild-type activities of the four promoter fusion proteins measured in 14 tester strains. Absolute β -galactosidase activities recorded for the wild-type background (Table 3) revealed that *flaA* has by far the strongest promoter exceeding the *flaB* and *flaC* promoter activities 6-fold and the *flaD* promoter activity nearly 100-fold.

Ideally, the ratio of mutant to wild-type activity is 1 if the mutant allele exerts no control over the tested promoter, and it is close to 0 if the native allele is needed for gene expression. Accordingly, the data listed in Table 3 led to three conclusions.

(i) Transcriptional control is exerted entirely on *flaA* promoter activity, whereas the *flaB*, *flaC*, and *flaD* promoters are not affected. (ii) *flaA* transcription is severely reduced (between 70 and 80%) whenever a nonfunctional flagellin combination is synthesized. This finding is consistent with the flagellin levels estimated by Western blot analyses (Fig. 5) and listed in Table 3. (iii) *flaA* promoter activity increases and approaches wild-type levels in parallel with the quality of the functional subunit combinations, i.e., with the amount and number of secondary Fla subunits in combination with the principal FlaA protein. (iv) Any restraint on transcription is suspended when no flagellin is synthesized ($\Delta flaABCD$), suggesting that it is the abortive flagellin (obviously not exported) that mediates transcriptional down-regulation of *flaA*. Taken together, the data point to an effective transcriptional control of the principal flagellin gene, *flaA*, exerted by nonfunctional flagellin subunit combinations.

DISCUSSION

In an attempt to relate the structure of the complex flagellar filaments of *R. lupini* and *S. meliloti* to their subunit compositions, we have used mutational analysis to study the contributions of the various flagellins to filament structure and to the regulation of flagellin biosynthesis. Of the three (*R. lupini*) or four (*S. meliloti*) flagellins, one (FlaA) is the principal subunit, while the others (FlaB, FlaC, and FlaD), though very similar, are accessory subunits. It takes a combination of FlaA and at least one accessory flagellin to assemble a functional filament. It is also the transcription of the principal FlaA-encoding gene (*flaA*) that is down-regulated whenever a nonproductive combination of Fla proteins prevents the assemblage of a func-

FIG. 4. Electron micrographs of *R. lupini* and *S. meliloti* wild-type and mutant flagellar filaments negatively stained with uranyl acetate. Wild-type *R. lupini* (A) and *S. meliloti* (D) complex flagellar filaments are dominated by a prominent three-start helical band pattern (zigzag pattern). Mutant filaments lacking the FlaA subunit from either *R. lupini* (B) or *S. meliloti* (E) are shown. Their complex surface structures appear to be normal (C and F). Bars: 100 nm (A, C, D, and F) and 500 nm (B and E).

tional filament. This type of control adds a new twist to the regulatory devices operating in bacterial flagellar synthesis.

The complex filaments of *R. lupini* H13-3 and, by analogy, those of *S. meliloti* differ from plain enterobacterial filaments

by a pairwise perturbation of the helical lattice, resulting in a subunit composed of a dimer of flagellin (38, 39). This earlier observation has been related to the presence of several distinct flagellin subunits in the complex filament by the proposal of a

FIG. 5. Immunoblot analysis of flagellin subunit proteins present in wild-type and mutant strains of *R. lupini* (A) and *S. meliloti* (B). Equal amounts of total cell protein (of ca. 10^7 cells contained in 20 μ l at an OD_{600} of 0.3) of each strain listed were separated by denaturing gel electrophoresis, blotted on a nitrocellulose membrane, and detected with polyclonal antibody raised against purified flagella from *R. lupini* (A) and *S. meliloti* (B), respectively. Approximate band intensities are listed in Table 3.

TABLE 3. Transcription of four *fla* promoters fused to *lacZ* in wild-type and *fla* mutant tester strains of *S. meliloti*

Genotype of tester strain	Relative promoter activity assessed with <i>lacZ</i> fusions in ^a :				Fla protein ^b
	pRU2274 (<i>flaA-lacZ</i>)	pRU2275 (<i>flaB-lacZ</i>)	pRU2276 (<i>flaC-lacZ</i>)	pRU2277 (<i>flaD-lacZ</i>)	
Wild type	1.00 (14,000)	1.00 (2,400)	1.00 (2,200)	1.00 (160)	++
Δ <i>flaA</i>	0.26	1.04	1.06	1.14	+
Δ <i>flaB</i>	0.77	0.98	1.06	0.98	++
Δ <i>flaC</i>	1.11	1.00	1.02	0.90	++
Δ <i>flaD</i>	1.05	0.97	1.01	0.98	++
Δ <i>flaAB</i>	0.22	1.00	0.99	1.07	(+)
Δ <i>flaAC</i>	0.23	0.97	1.08	0.96	(+)
Δ <i>flaBC</i>	0.78	1.04	1.04	0.95	++
Δ <i>flaBD</i>	0.71	0.98	1.05	1.03	++
Δ <i>flaCD</i>	0.67	0.99	0.95	0.81	++
Δ <i>flaABC</i>	0.25	0.99	1.03	1.03	ND ^c
Δ <i>flaABD</i>	0.24	1.05	0.96	0.95	(+)
Δ <i>flaACD</i>	0.22	1.04	1.05	0.85	(+)
Δ <i>flaBCD</i>	0.33	1.03	0.98	0.83	(+)
Δ <i>flaABCD</i>	1.12	1.08	1.07	0.71	–

^a Relative activities are expressed as ratios of β -galactosidase activity in the indicated mutant backgrounds to that in the wild-type background. Transcription from four *fla* promoters was assessed with plasmid-borne *lacZ* fusions (see Materials and Methods). Mean values of Miller assays (24) were averaged, with standard deviations ranging between 0.01 and 0.15. Values in parentheses are Miller units of β -galactosidase activity (24) averaged from the results of three independent assays. The reduced *flaA* promoter activities are highlighted by boldface numbers.

^b Approximate amounts of flagellin deduced from immunoblot (Fig. 5) band intensities. ++, normal; +, reduced; (+), severely reduced; –, zero.

^c ND, not determined.

structure composed of flagellin heterodimers (29). This concept has been strengthened by the present finding of a principal FlaA and several secondary FlaX subunits that together constitute a functional filament. In support of a heterodimer model is the need for a combination of FlaA and at least one accessory flagellin for assembling a functional filament. This assembly requires a FlaA-FlaX stoichiometry of 1:1. However, when we compared *flaA* promoter activity to the combined activities of the *flaB*, *flaC*, and *flaD* (= *flaX*) promoters (Table 3), the derived *flaA*-to-*flaX* transcript ratio was about 3:1, suggesting that, on average, only every second dimer in the filament was a FlaA-FlaX heterodimer. On the other hand, an inspection of band intensities on immunoblots (Fig. 5) suggested that, upon translation, FlaA and the secondary FlaX proteins result in a 1:1 ratio. This becomes particularly obvious in *R. lupini*, whose FlaA and FlaB proteins migrate at different mobilities (Fig. 5A). Also, in *S. meliloti*, a balanced FlaA-FlaX stoichiometry is evidenced by the Δ *flaA* mutant which reveals roughly half the FlaX band intensity seen in the heavy single band of the wild-type strain (Fig. 5B). Such a balanced stoichiometry does, in fact, indicate a heterodimeric FlaA-FlaX substructure of the complex filament. It also implies an as yet unknown translational control of flagellin biosynthesis that equalizes the dominance of *flaA* transcription.

The transcription of the single flagellin gene, *fliC*, of *E. coli* proceeds from a particular promoter sequence, recognized by a specific sigma factor, σ^{28} , encoded by *fliA*. This class II gene (20) is part of the hierarchical flagellar control system, expressed only when flagellar basal-body components are already being assembled. The *fliA*-encoded σ^{28} itself is antagonized by a specific anti- σ^{28} factor, FlgM, which is eventually removed from the cytoplasm by export through the completed flagellar

channel. FliA, which remains behind, is now capable of binding to the flagellin promoters. As reported here, the complex ribzobial filaments differ from plain *E. coli* filaments in their compositions by containing more than one type of flagellin. They also differ in the negative transcriptional control of *flaA* exerted by abortive combinations of Fla subunits that regulate FlaA biosynthesis in addition to the global control by VisNR (35). Can we envision a regulatory mechanism consistent with the current results? Upstream of the *R. lupini* and *S. meliloti* *fla* genes are sequences (delineated in Fig. 6) that resemble the σ^{28} consensus sequence derived for *Vibrio parahaemolyticus* (23) and the enterobacterial flagellar promoters (11). As previously elaborated by primer extension analysis of the *flaA* and *flaB* mRNAs of *S. meliloti* (28), these σ^{28} -responsive elements represent the most likely candidates for *fla* promoters. Very similar promoter elements have been detected by sequence alignments upstream of all *R. lupini* and *S. meliloti* *fla* genes (Fig. 6). Although the –35 sequences of all seven *fla* genes are perfectly conserved, the –10 sequences deteriorate from *flaA* to *flaD* in comparison to the consensus sequences. Diminishing promoter quality is seen as the main reason for the decrease in transcriptional activity from *flaA* to *flaD*, as listed in Table 3.

A plausible model for the unusual down-regulation of *flaA* transcription reported here postulates jamming of the export channel by unproductive combinations of flagellins (missing the FlaA or the FlaX subunits) that cannot be assembled at the distal end. Rather than postulating a direct inhibitory interaction of excess flagellin accumulated in the cell with *flaA* transcription, we think that an FlgM-like anti- σ^{28} protein (yet to be identified) is being prevented from escaping and, doomed to remain inside the cell, continues to render σ^{28} -dependent transcription from the primary *flaA* promoter inactive. In order to substantiate this model, we are presently screening for mutants able to overcome the down-regulation of *flaA*. One may raise the objection that, different from the data of Table 3, an anti- σ^{28} device should affect all four *fla* genes of *S. meliloti*, given

	–35		–10
Rl <i>flaA</i> *	TTAc.aA	N _{1,3}	GCgGtaAc
Rl <i>flaA</i>	TTAA.aA	N _{1,4}	GCTGCCAA
Rl <i>flaB</i>	TTAA.CA	N _{1,4}	aagGCCAt
Rl <i>flaD</i>	TTAA.GA	N _{1,4}	GCaGgtcg
Sm <i>flaA</i> *	TTAA.CA	N _{1,3}	GCgattAA
Sm <i>flaA</i>	TTAA.CA	N _{1,4}	GCTGCgAA
Sm <i>flaB</i>	TTAA.CA	N _{1,4}	agacCCAt
Sm <i>flaC</i>	TTAA.CA	N _{1,4}	tCgttaAg
Sm <i>flaD</i>	TTAA.CA	N _{1,4}	GggetCAA
Rl/Sm con	TTAA.CA	N _{1,4}	GCTGCCAA
VPA con	TAAA	N _{1,5}	GCCGTTAA
σ^{28}	TAAA	N _{1,5}	GCCGATAA

FIG. 6. DNA sequence alignment of putative promoter sequences upstream of the *R. lupini* (Rl) and *S. meliloti* (Sm) *fla* genes. *flaA** denotes a secondary promoter sequence further upstream from the *flaA* gene (28). Capital letters indicate homology with consensus residues at a given alignment position. The *R. lupini* and *S. meliloti* (Rl/Sm) flagellin promoter consensus sequence was derived from a minimum plurality of four matches (except for position 3 of the –10 box, which was adapted to the highly active *flaA* promoter sequences). The consensus sequences of *V. parahaemolyticus* polar flagellar (VPA) (23) and *E. coli* σ^{28} (11) promoters are shown for comparison.

their transcription from σ^{28} -specific promoters. In principal, this is true. However, even at a maximum reduction to about 20%, *flaA* transcription still runs at a level above that of the standard transcription of *flaB*, *flaC*, or *flaD* (Table 3). It may be noted that there are secondary *flaA* promoter motifs located further upstream of the strong *flaA** (28) promoters of both *R. lupini* and *S. meliloti* (Fig. 6); these may well account for the residual 20% transcription. We thus infer that down-regulation by nonassembled flagellin affects and reduces high-expression *flaA* but does not affect the secondary *flaA** promoters and those of the lowly transcribed *flaX* genes.

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