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Analysis of the *Erwinia chrysanthemi arb* Genes, Which Mediate Metabolism of Aromatic β-Glucosides

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***Erwinia chrysanthemi* is one of the few members of the family *Enterobacteriaceae* that is capable of metabolizing most of the naturally occurring β-glucosides. We previously isolated the *clb* genes, which allow the use of the disaccharide cellobiose as well as the aromatic β-glucosides arbutin and salicin. We report here the isolation of the *arb* genes, which permit fermentation of the aromatic β-glucosides only. Establishment of a functional Arb system in *Escherichia coli* depended on the presence of the phosphotransferase system and on the activation by the cyclic AMP-cyclic AMP receptor protein complex. Strains carrying mini-Mu-induced LacZ fusions to the *arb* genes were used to analyze *arb* genes organization and function. Three *arb* genes (*arbG*, *arbF*, and *arbB*) were identified and organized in this order. Genetic and structural evidence allowed us to assign a phospho-β-glucosidase and a permease activity to the ArbB and ArbF proteins, respectively. Several Lac$^+$ *arb-lacZ* insertions were introduced into the *E. chrysanthemi* chromosome. Both ArbG$^-$ and ArbF$^-$ strains were unable to ferment the aromatic β-glucosides, whereas ArbB$^-$ strains were impaired only in salicin fermentation. None of the mutations in the *arb* genes affected cellobiose metabolism. The expression of the *arb* genes was substrate inducible and required the ArbF permease and, possibly, the ArbG protein. Collectively, our results underline the resemblance between the naturally expressed *E. chrysanthemi arbGFB* and the cryptic *E. coli bglGFB* operons, yet the *arbG* gene product seemed unable to activate *E. coli bgl* operon expression.**

Bacterial species from the family *Enterobacteriaceae* differ in their capacity to metabolize the β-glucosides cellobiose, salicin, arbutin, and esculin (27). In recent years, the catabolic pathways of those compounds have been studied in *Escherichia coli* (1, 13, 28) and, to a lesser extent, in *Erwinia chrysanthemi*, a phytopathogenic bacterium (4, 8).

E. chrysanthemi is capable of using arbutin, salicin, and cellobiose as carbon sources. This is of special interest because these β-glucosides are natural constituents of plant tissues and *E. chrysanthemi* causes the so-called soft-rot disease of numerous dicotyledonous plants (8). We previously isolated the *clb* genes of *E. chrysanthemi* (3). Biochemical and genetic characterization of the *clb* system revealed the following: (i) it allows *E. coli* to use cellobiose, arbutin, and salicin (6); (ii) β-glucoside uptake is mediated through the phosphotransferase system (PTS) in *E. coli* (5) and *E. chrysanthemi* (9); and (iii) *clb* genes are present as three transcriptional units, an atypical organization within the context of PTS-dependent catabolic systems (4). Further work showed that marker-exchanged *clb* mutants of *E. chrysanthemi* failed to use cellobiose but retained the capacity to use arbutin and salicin (J. P. Chambost, unpublished data). The simplest interpretation of these data was that in *E. chrysanthemi* there are two systems that exhibit overlapping specificities.

Although *E. coli* K-12 is naturally unable to use any of the β-glucosides, it has four cryptic systems that, when activated, allow the use of some β-glucosides (20, 22, 23). The best-characterized system is the *bglGFB* operon. The *bglF* and *bglB* gene products allow the uptake and the hydrolysis, respectively, of the aromatic β-glucosides arbutin and salicin (15, 29). The BglF protein is a typical PTS-specific enzyme II (EII) that receives a phosphate group from the general PTS enzymes EI and HPr (25) and transfers that group to the sugar while catalyzing its entry into the cells. In the presence of aromatic β-glucosides, BglG protein acts as a positive effector of the *bgl* operon expression through a complex antitermination mechanism (1, 15, 16, 28). In the absence of β-glucosides, BglF acts as a kinase of BglG, and the resulting phosphorylated form is unable to activate *bgl* operon expression. The incoming phospho-β-glucoside is then hydrolyzed by the BglB protein. A second PTS-dependent system, located opposite the *bgl* operon on the chromosomal map and refered to as the *cel* operon, allows the utilization of arbutin, salicin, and cellobiose (13, 20). The question of the affinity of either one of these two systems for esculin was not addressed.

In this study we isolated the *E. chrysanthemi arb* system, which mediates arbutin and salicin metabolism. Its genetic regulation and organization were determined and compared with those of the *E. chrysanthemi clb* and *E. coli bgl* systems. Both *E. coli bgl* and *E. chrysanthemi arb* systems allowed the fermentation of the fourth naturally occurring β-glucoside, esculin.

MATERIALS AND METHODS

Bacteria and plasmids. Bacterial strains, bacteriophages, and plasmids used in this work are listed in Table 1.

Media and chemicals. Bacterial strains were grown either in L or M9 medium (19) at 32°C for *E. chrysanthemi* and at 37°C for *E. coli*. When required, the medium contained ampicillin (50 μg/ml), tetracycline (25 μg/ml), streptomycin (25 μg/ml), or chloramphenicol (25 μg/ml). Carbon sources were added at 0.4%. MacConkey plates containing appropriate sugars (0.4%) were used for fermentation tests. When added as inducers, arbutin and salicin were present at 7 mM and α-methyl-β-D-glucoside (MBG) was at 10 mM.

Molecular cloning. The genomic library of *E. chrysanthemi* 3665 was constructed by standard procedures (17). Briefly,

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TABLE 1. Bacterial strains, bacteriophages, and plasmids

Strain, phage, or plasmid	Genotype	Source
E. coli		
MC1061	*araD139* Δ(*ara-leu*)*7697* Δ*lacX74* *galU galK hsdR rpsL thi*	M. Casadaban
MC4100	F⁻ Δ*lacU169 araD139 rpsL thiA relA*	M. Casadaban
MC4100-B	As MC4100 with *pcnB80 zad*::Tn*10*	This work
PR13	As MC4100 and Mu dIIPR13	F. Richaud
DG37	Δ(*ptsIH-cys*) *thi relA1*	A. Danchin (11, 12)
TP2505	*crr xyl argH1 ilvA*	A. Danchin
RH5558	Δ*cya his xyl*	A. Danchin
RH5558-B	Δ*cya his xyl pcnB80* Tn*10*	This work
CH208	*gal araD139* Δ*lacU169 zab*::Tn*5* *rpsL* Δ*crp*	V. A. Bankaitis
MRi84	As MC4100 and Δ*rbs-7 pcnB80 zad*::Tn*10*	J. Beckwith (14)
AE304-1	F⁻ Δ*lacX74 thi bglR11 tsx* T6ʳ *tna*::Tn*10 bglF2*	A. Wright
AE304-3	F⁻ Δ*lacX74 thi bglR11 tsx* T6ʳ *tna*::Tn*10 bglB3*(Am)	A Wright
AE304-9	F⁻ Δ*lacX74 thi bglR11 tsx* T6ʳ *tna*::Tn*10 bglG8*	A. Wright
E. chrysanthemi		
3665	Wild type	M. Lemattre
EH363	As 3665 and *arb-363*::Mu dII PR13	This work
EH344	As 3665 and *arb-344*::Mu dII PR13	This work
EH334	As 3665 and *arb-334*::Mu dII PR13	This work
Bacteriophages		
Mu dII PR13	Mu *cts* 62 dII(Cmʳ, *lac'ZYA*) with Δ(Mu AB)*Hin*dIII	F. Richaud
P1	P1*vir*	Laboratory collection
Plasmids		
pBR322	Apʳ Tʳ ColE1 derivative	
pEM31	*arbG arbF arbB*, Apʳ	This work
pBH363	*arbG363*, Lac⁺ Apʳ Cmʳ	This work
pBH344	*arbF344*, Lac⁺ Apʳ Cmʳ	This work
pBH334	*arbB334*, Lac⁺ Apʳ Cmʳ	This work

total chromosomal DNA was extracted as described by Marmur (18) and partially digested with *Sau*3A, and fragments were sized on a sucrose gradient. Fragments were inserted into the unphosphorylated *Bam*HI restriction site of pBR322. Recombinant plasmids were isolated by transforming *E. coli* MC1061, selecting for Apʳ, and scoring for Tcˢ clones. All further DNA manipulations were carried out by standard methods (17). Enzymes were purchased commercially and used as recommended by the manufacturer.

Genetic techniques. Transformation of *E. coli* strains was performed after treating the cells with 10% dimethyl sulfoxide–5% polyethylene glycol–20 mM Mg^{2+} (10). P1 lysates were made by standard methods (19). Insertions of mini-Mu elements in plasmid DNA were performed as described by Castilho et al. (7). Briefly, thermoinduction of the *E. coli* PR13(pEM31) strain was performed for 1 h at 42°C, and the resulting lysate was used to infect a Mu lysogenic derivative of MC4100 for 1 h at room temperature. Transductants harboring mutagenized pEM31 plasmids were selected as Cmʳ Apʳ.

***p*-Nitrophenyl-β-D-glucoside plate assays.** The *E. coli* strain containing the pEM31 plasmid was grown on minimal medium supplemented with succinate (0.4%) as the carbon source and salicin or MBG (0.002%) as the inducer. A sample of ca. 50 μl from a solution (0.4 mM) of *p*-nitrophenyl-β-D-glucoside was placed on the colonies. The times required for yellow color to develop in cells grown with and without inducers were compared.

Measurement of enzymatic activities. β-Galactosidase activity was assayed as described by Miller (19), and phospho-β-glucosidase activity was assayed as described by Schnetz and Rak (28). One unit of phospho-β-glucosidase activity is defined by the amount of *p*-nitrophenol released per unit of optical density at 600 nm of cell suspension per minute. Experiments were repeated at least four times, starting from independent bacterial cultures.

Marker-exchange mutagenesis in *E. chrysanthemi*. Plasmids carrying mutated *arb* alleles were introduced into the *E. chrysanthemi* strains by electroporation with a Bio-Rad unit (2.5 kV, 25 μF, 200 Ω). Cells were treated as recommended for the preparation of *E. coli* cells (2). After several subcultures in L medium without antibiotic, the last culture was grown in the presence of chloramphenicol, plated onto rich medium with chloramphenicol, and replica plated on rich medium with ampicillin. The Cmʳ Apˢ colonies were saved for further studies.

Western blotting analysis of hybrid proteins. Cultures were grown in L medium to an optical density at 600 nm of 1.0. A sample of 0.5 ml was centrifuged for 2 min, and the pellets were suspended in 50 μl of water and 20 μl of buffer (20 mM Tris hydrochloride [pH 8.8], 5 mM EDTA, 1 M sucrose, 18% sodium dodecyl sulfate, 0.3 M dithiothreitol, 5% bromophenol blue). Samples were heated to 95°C for 10 min, returned to ice, and stored at −20°C. Protein content was analyzed with a homogenous 7% sodium dodecyl sulfate–polyacrylamide gel run on a Pharmacia Phast System unit. Proteins containing a β-galactosidase moiety were visualized after the gel was transferred to nitrocellulose paper and blotted against mouse anti-β-galactosidase. After washing, alkaline phosphatase-conjugated anti-mouse immunoglobulin was added, and the nitrocellulose paper was developed with the alkaline phosphatase activity indicators 5-bromo-4-chloro-3-indolylphosphate and Nitro Blue Tetrazolium.

RESULTS

Isolation of the *arb* genes. A genomic bank of *E. chrysanthemi* 3665 was constructed in *E. coli*. Screening on MacConkey plates supplemented with arbutin led to the identification of one Arb⁺ clone that was also capable of fermenting salicin but not cellobiose. Tests for the use of these sugars as carbon sources in minimal medium gave the same pattern. The recombinant plasmid pEM31 was isolated and used as a probe for analyzing the DNA homology level shared with the *E. chrysanthemi clb* genes; no hybridization was observed (data not shown). These results indicated that the pEM31 plasmid harbors a new set of *E. chrysanthemi* genes, hereafter referred to as *arb* genes, encoding proteins involved in aromatic β-glucoside metabolism.

Requirements for *E. chrysanthemi* Arb system expression in *E. coli*. We used various well-defined *E. coli* mutant strains to investigate *E. chrysanthemi arb* gene expression in *E. coli*. Plasmid pEM31 was introduced into *E. coli* DG37, which is devoid of the general PTS enzymes EI and HPr. The resulting strain was not capable of fermenting either arbutin or salicin on MacConkey plates (Table 2). Therefore, the

TABLE 2. Phenotype on MacConkey arbutin (or salicin) medium of *E. coli* strains carrying the pEM31 plasmid[a]

Strains	Relevant genotype	Colony color
MC4100	*arbGFB*$^+$	Red
MC4100-B	*pcnB arbGFB*$^+$	Pink
DG37	Δ(*ptsIH-cys*) *arbGFB*$^+$	White
TP2505	*crr arbGFB*$^+$	Red
CH208	Δ*crp arbGFB*$^+$	Red
RH5558	Δ*cya arbGFB*$^+$	Red
RH5558-B	Δ*cya arbGFB*$^+$ *pcnB*	White
RH5558-B	Δ*cya arbGFB*$^+$ *pcnB*	Red[b]

[a] Strains were incubated for 24 h at 37°C.
[b] cAMP (5 mM) was added to the medium.

Arb system requires a functional PTS, and this is likely to involve an EII-like enzyme for β-glucoside uptake.

Similarly, we tested the involvement of the specific $EIII^{Glc}$ enzyme by introducing plasmid pEM31 into *E. coli* TP2505. The resulting transformant was capable of fermenting both β-glucosides (Table 2), thereby ruling out a role for the $EIII^{Glc}$ enzyme in this metabolism.

Next, we assessed the role of the cyclic AMP (cAMP)-cAMP receptor protein (CRP) complex in *arb* gene expression. Plasmid pEM31 was introduced into *E. coli* RH5558, which is devoid of adenylate cyclase activity, and *E. coli* CH208, which is impaired in catabolite activator protein synthesis. Ambiguous data were obtained; although at a reduced rate, both *E. coli* RH5558(pEM31) and CH208 (pEM31) were capable of fermenting arbutin or salicin (Table 2). A possibility was that the presence of multiple copies of the *arb* genes was overcoming the absence of cAMP-CRP activation. Therefore, the *pcnB* mutation that reduces the copy number of ColE1-type plasmids was transduced into both *E. coli* RH5558(pEM31) and CH208(pEM31). The decrease in plasmid copy number was estimated by comparing the β-lactamase activity levels in both parental and $PcnB^-$ strains. The pEM31 plasmid was present at 30 and 3 copies per cell in the parental and $PcnB^-$ strains, respectively (data not shown). The transductants were unable to ferment the β-glucosides, unless cAMP was exogenously added (Table 2). This indicated that *arb* gene expression is indeed under cAMP-CRP complex-mediated positive control. The fact that the lack of this control can be compensated for by increased gene dosage might mean either that this control is slightly leaky or that *arb* genes on plasmid pEM31 can be expressed from a second cAMP-CRP-independent weak promoter.

Finally, we asked whether a phospho-β-glucosidase activity was encoded by the pEM31 *arb* genes. Such an enzymatic activity was indeed revealed by using the semiquantitative *p*-nitrophenyl-β-D-glucoside test (see Materials and Methods). Furthermore, in the presence of salicin or MBG, a nonmetabolizable β-glucoside analog, the yellow color developed much more rapidly than without these compounds, suggesting that the phospho-β-glucosidase synthesis was substrate inducible. This was confirmed by directly assaying phospho-β-glucosidase activity; the induction ratio was at least 5. In strain MC4100(pEM31), the phospho-β-glucosidase activities were 15 and 16.5 U with arbutin and salicin (7 mM), respectively, and 3.3 U without an inducer; all values for strain MC4100 were <1 U.

Genetic organization of the *arb* genes. A detailed restriction map of pEM31 showed that it carried an insert of approximately 7.3 kb (Fig. 1). Plasmid pEM31 was then subjected to insertion mutagenesis with mini-Mu-*lacZ*-forming fusion element PR13 (7). Colonies carrying mutagenized plasmids were selected using the Cm^r marker carried by the PR13 element and were subsequently tested for their capacities to ferment β-glucoside. Colonies fell into three phenotypically distinct groups: Arb^- Sal^-, Arb^+ Sal^-, and Arb^+ Sal^+. The second group, Arb^+ Sal^-, was heterogeneous; on MacConkey arbutin plates some colonies were deep red, whereas others only had a red center. The insertions present in the plasmids of the first two groups mapped throughout a 4.3-kb region (Fig. 1). Moreover, we noted that the insertions in the Arb^+ Sal^- group mapped toward one end of this 4.3-kb region. This region was flanked by the insertions found in the third group of plasmids, i.e., the Arb^+ Sal^+ group (Fig. 1).

The PR13 element carries a *lacZ* gene (encoding the *E. coli* β-galactosidase) that is devoid of both transcriptional and translational initiation signals. Hence, if the element inserts in frame, hybrid β-galactosidase protein is produced in which the NH_2 terminus is provided by the targeted gene (7). All plasmids carrying an inserted PR13 element were scored for their Lac phenotype on MacConkey-lactose plates. Three Lac^+ insertions, 324, 363, and 344, were found among the plasmids of the Arb^- Sal^- group. Three other Lac^+ insertions, 346, 301, and 334, respectively, were found

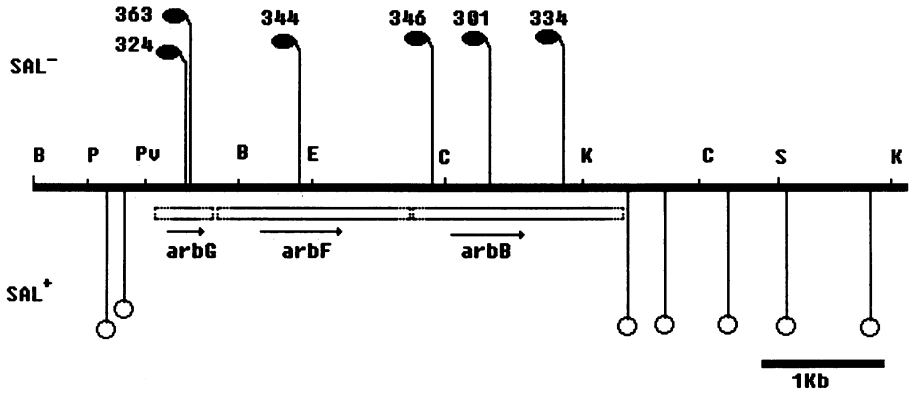

FIG. 1. Genetic organization of the *arb* genes. The middle dark bar represents the chromosomal insert cloned on the pEM31 plasmid, including the location of restriction sites. The closed circles represent mini-Mu insertions that lead to a Sal^- Lac^+ phenotype, whereas the open circles represent the Sal^+ insertions. Tentative locations of the *arb* gene endpoints are proposed. Abbreviations: B, *Bam*HI; P, *Pst*I; Pv, *Pvu*II; E, *Eco*RI; C, *Cla*I; K, *Kpn*I; S, *Sal*I.

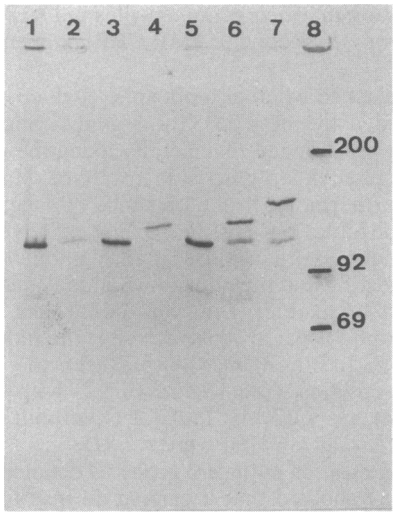

FIG. 2. Western blotting analysis of crude cell lysates of *E. coli* producing various Φ(*arb-lacZ*) hybrid proteins. Same amounts of cells were loaded in each lane. Hybrid proteins containing β-galactosidase were revealed by using commercially prepared antiserum anti-β-galactosidase. Lanes: 1, β-galactosidase; 2, Arb-LacZ324; 3, Arb-LacZ363; 4, Arb-LacZ344; 5, Arb-LacZ346; 6, Arb-LacZ301; 7, Arb-LacZ334; 8, marker size.

among the plasmids of the Arb^+ Sal^- group. On MacConkey-arbutin plates, the clones containing fusion 334 gave deep red colonies, whereas the clones containing fusions 346 and 301 appeared as white colonies with red centers. The orientation of each inserted PR13 element leading to Lac^+ colonies was further determined by restriction analysis; all were in the same direction (Fig. 1). Hence it appeared that the cloned insert carries several clustered *arb* genes that are all transcribed in the same direction.

Analysis of Φ(*arb-lacZ*)-encoded hybrid proteins. There should be a correlation between the size of the encoded hybrid protein and the location of the fusion insertion in the *arb* gene relative to its cognate translational initiation codon. Hence, knowledge of both the insertion locations and the hybrid protein sizes should provide us with some information concerning the numbers and the lengths of the *arb* genes. Location of the insertion points was estimated by restriction analysis. The unique *Pvu*II restriction site of the insert was taken as an arbitrary origin, since it is located at the limit between the first (Arb^- Sal^-) and the third (Arb^+ Sal^+) groups of pEM31::PR13 plasmids (Fig. 1). The sizes of the Φ(*arb-lacZ*)-encoded hybrid proteins were obtained by Western blotting against anti-β-galactosidase antiserum (Fig. 2). Fusion 324 and 363 insertion points mapped 0.3 kb from the *Pvu*II site. The cognate hybrid proteins had an M_r of 120,000. Taking into account the size of the β-galactosidase moiety (M_r, 115,000), this implied that both hybrid proteins contained ca. 45 amino acids encoded by the targeted *arb* gene. In turn, this indicated that the insertion points of both fusions were located approximately 0.15 kb from the translational start codon, which was therefore assumed to be 0.15 kb downstream of the *Pvu*II restriction site. The insertion point of fusion 344 was 1.1 kb from the *Pvu*II restriction site, that is, 0.95 kb from the previously identified initiation codon. Hence, the encoded hybrid protein should contain 333 *arb*-encoded amino acids fused to the β-galactosidase, giving rise to a protein of M_r 152,000. This was not the case, since fusion 344 produced a hybrid protein of M_r 140,000, indicating the presence of approximately 225 *arb*-encoded amino acids at the NH_2 terminus. We concluded that fusion 344 inserted within a second *arb* gene, located immediately downstream from the gene inactivated by fusions 324 and 363. The initiator codon of that second gene would be ca. 0.4 kb from the *Pvu*II restriction site. Accordingly, the size of the first *arb* gene should not exceed 0.25 kb. Fusion 346 was located 2.3 kb downstream from the *Pvu*II restriction site, i.e., 1.9 kb downstream from the initiator codon of the second gene. If fusion 346 were located within that gene, the corresponding hybrid protein would be of M_r 185,000. This was not the case, since fusion 346 produced a protein of M_r 120,000, i.e., contained approximately 45 *arb*-encoded amino acids fused at the NH_2 terminus of the β-galactosidase. Therefore, we concluded that fusion 346 mapped at ca. 0.15 kb downstream from the translational initiation codon of a third *arb* gene. Accordingly, the size of the second *arb* gene should be ca. 1.7 kb. Insertion points of fusions 301 and 334 were located 0.5 and 1.2 kb from the fusion 346 insertion point, respectively. Therefore, if both fusions 301 and 334 were located within the third *arb* gene, the corresponding hybrid proteins should contain approximately 216 and 450 amino acids fused to the β-galactosidase, respectively, thereby giving rise to hybrid proteins of approximately M_r 139,000 and 164,000, respectively. These predicted values were consistent with those experimentally obtained; fusions 301 and 334 produced hybrid proteins of M_r 134,000 and 156,000, respectively. Taken together, these data supported the view that three *arb* genes occur in the cluster; these are referred to as *arbG* (0.25 kb), *arbF* (1.7 kb), and *arbB* (>1.8 kb) (Fig. 1). Moreover, it is noteworthy that the amount of hybrid proteins produced varied from one fusion to the other, as visualized by immunoblotting. In particular, the amount of the fusion 324 was much lower than that produced by the fusion 363. Differential protein stability or translational efficiency might be the cause, but the molecular basis for this difference was not investigated.

Construction of *E. chrysanthemi arb* mutants. To study the functioning of the *arb* genes in their normal context, Φ(*arbG-lacZ*)*363*, Φ(*arbF-lacZ*)*344*, and Φ(*arbB-lacZ*)*334* were introduced into the *E. chrysanthemi* chromosome. To do this, plasmids pBH363, pBH344, and pBH334 were electroporated within *E. chrysanthemi*. Taking advantage of the instability of ColE1-type plasmids in *E. chrysanthemi*, we subcultured the transformants in rich medium without antibiotic selection. Colonies were then plated onto rich medium supplemented with chloramphenicol, thereby selecting those cells that either had retained the plasmid or had undergone a reciprocal recombination event between the chromosomal *arb* and the Φ(*arb-lacZ*) plasmid copy. Colonies of the latter were subsequently identified by their sensitivity to ampicillin. Three strains, (EH363, EH344, and EH334) were isolated that contained the *arbG363*, *arbF344*, and *arbB334* mutations, respectively. The three mutants were analyzed for their ability to use arbutin, salicin, or cellobiose as a carbon source on minimal medium (Table 3). Both strains EH363 and EH344 were Clb^+ Arb^- Sal^-, whereas strain EH334 was Clb^+ Arb^+ Sal^-. In fermentation tests, the same phenotypes were observed. These results clearly established the existence of two separate β-glucoside assimilatory pathways in *E. chrysanthemi*, i.e., pathways controlled by *arb* and *clb*.

Analysis of the regulation of the *arb* genes in *E. chrysanthemi*. *E. chrysanthemi* EH363, EH344, and EH334 were used to analyze the regulation of β-galactosidase synthesis directed from the *arb* gene expression signals. Induced and

TABLE 3. Characteristics of *E. chrysanthemi* strains containing *arb* mutations

Strain	Phenotype on minimal medium[a] with:			β-Galactosidase activity[b] (Miller U)	
	Arbutin	Salicin	Cellobiose	−MBG	+MBG
3665	+	+	+	28	30
EH363	−	−	+	550	580
EH363(pEM31)	+	+	+	200	220
EH344	−	−	+	100	110
EH344(pEM31)	+	+	+	70	260
EH334	+	−	+	50	1,200
EH334(pEM31)	+	+	+	80	2,400

[a] β-Glucosides (0,4%) was used as the carbon source.
[b] MBG (10 mM) was used as the inducer. The data are averages of four experiments.

noninduced levels of β-galactosidase activity found in strains containing fusions Φ(*arbG-lacZ*)*363* and Φ(*arbF-lacZ*)*344* were identical (Table 3). In contrast, the β-galactosidase activity produced from fusion Φ(*arbB-lacZ*)*334* was highly inducible by MBG. This last result implied either that the *arbB* gene is expressed independently from *arbG* and *arbF* or that the latter two are required for positively controlling *arb* cluster expression. To test the first possibility, pEM31 derivative plasmids containing Mu insertions within *arbG* (e.g., pBH363) or *arbF* (e.g., pBH344) were transferred into *E. coli* AE304-3, which is devoid of phospho-β-glucosidase activity but retains permease functions. Both types of transformants were phenotypically Sal$^-$, indicating that no phospho-β-glucosidase was produced. Hence, the insertions in either *arbG* or *arbF* genes are polar on *arbB* expression, indicating that the expression of *arbB* was not independent of that of the two other *arb* genes. The possibility that the *arbG* and/or *arbF* gene product possesses a regulatory function was investigated by looking at the effect exerted by multiple copies of these genes upon the expression of the three *lacZ* fusions (Table 3). First, the presence of pEM31 plasmid led to a slight decrease in Φ(*arbG-lacZ*)*363* fusion expression, which remained noninducible. Second, the expression of the Φ(*arbB-lacZ*)*334* fusion was inducible, and the ratio of induction was higher than in presence of single copies of *arbG* and *arbF* genes (strain EH334). Third, the expression of the Φ(*arbF-lacZ*)*344* fusion was substrate inducible in the presence of pEM31, whereas it was not substrate inducible when the *arbF* gene was inactivated (strain EH344). Taken together, these data supported the idea that *arbF* at least, and possibly *arbG*, is required for β-glucoside-mediated induction (see Discussion).

Lack of complementation of the *E. coli bglG* mutation by *E. chrysanthemi arb* genes. As described above, the *E. coli bglG* gene plays a crucial role in the regulation of the *bgl* operon. Since the *E. chrysanthemi arb* cluster also appears to contain a positive effector, we wished to determine whether it could substitute for the *E. coli bglG* gene by positively regulating *bgl* operon expression. Plasmids pBH344 and pBH334 (carrying insertions in the *arbF* and *arbB* genes, respectively) were transferred to the Sal$^-$ *E. coli* AE304-9 strain. When tested on MacConkey plates, both types of transformants still exhibited the Sal$^-$ phenotype, indicating that no complementation had occurred.

Metabolism of esculin in *E. coli* and *E. chrysanthemi*. As previously mentioned, fermentation of the fourth naturally occurring β-glucoside, esculin, has been overlooked in genetic studies on β-glucoside metabolism in *E. coli*. Therefore, we decided to test various *E. coli* and *E. chrysanthemi* strains on MacConkey plates supplemented with esculin. Again, *E. coli* and *E. chrysanthemi* wild-type strains differed; only the latter was capable of fermenting esculin. Results with *E. chrysanthemi* EH363, EH344, and EH334 were ambiguous, since they ferment esculin at a reduced rate; the red colony coloration requires prolonged incubation to develop. Nevertheless, that the *arb* system was capable of catalyzing esculin metabolism was indicated by the fact that an *E. coli* strain bearing the pEM31 plasmid produced red colonies. In contrast, an *E. coli* strain expressing the *E. chrysanthemi clb* system did not ferment esculin. Interestingly, the *E. coli* mutant expressing the *bgl* operon was capable of fermenting esculin. The requirement for the *bgl* genes in that phenotype was demonstrated by the esculin-negative phenotype of AE304-3, AE304-9, and AE304-10 strains. Unexpectedly, neither *E. coli* strains expressing the *bgl* operon or carrying the pEM31 plasmid nor the *E. chrysanthemi* wild type was capable of using esculin as a carbon source when grown on minimal medium. Collectively, these observations highlighted the resemblance between the *E. chrysanthemi arb* and *E. coli bgl* systems and the difference between the *E. chrysanthemi clb* and *arb* systems.

DISCUSSION

Our previous studies suggested the occurrence of two PTS-dependent pathways allowing the use of β-glucosides in *E. chrysanthemi* (4, 9). The *clb* genes were found to control the metabolism of cellobiose as well as arbutin and salicin. The uptake pathway was induced by cellobiose only. In this paper we report the isolation of the genes encoding the second system, referred to as Arb, which allows the fermentation of arbutin, salicin, and esculin.

The study of the Arb system expression in *E. coli* confirmed the presence of both a PTS-dependent permease and a phospho-β-glucosidase. Similarly, the genetic analysis of *arb* gene expression indicated that both the β-glucosides and the cAMP-CRP complex act as activators. This last point was recently shown to be true in *E. chrysanthemi* by using a strain devoid of adenylate cyclase activity (P. Glaser and A. Danchin, unpublished data), which exhibited an Arb$^-$ Sal$^-$ phenotype.

Mini-Mu mutagenesis performed in *E. coli* revealed that the *arb* genes lie over 4.3 kb. By correlating the size of the hybrid proteins synthesized with the position of the respective Mu insertion element, we established the existence of three genes, *arbG*, *arbF*, and *arbB*. The phenotypic analysis of various mutants defective in each *arb* gene showed that all three are required for metabolizing salicin. In contrast, mutations in the *arbB* gene moderately affected (*arbB346*, *arbB301*) or did not change (*arbB334*) the fermentation rate of arbutin. In *E. coli*, this has to be connected with the constitutive production of the phospho-β-glucosidase A, which hydrolyzes the phosphoarbutin only (26). Likewise, the fact that *E. chrysanthemi* EH334 exhibited an Arb$^+$ Sal$^-$ phenotype strongly suggested the existence of a *bglA*-like gene in that bacterium. Collectively this implied that *arbB* encodes a phospho-β-glucosidase enzyme, whereas transport functions are encoded by *arbG* and/or *arbF*.

The use of *lacZ* fusions allowed us to characterize the genetic organisation of the *arb* genes. Although it was not demonstrated that the *arb* genes are present as a single transcription unit, the high linkage and the common transcriptional direction of all three genes and the apparently

polar effect of some insertions make this hypothesis likely (see below). From the size of the hybrid protein analysis, we deduced that the ArbG and ArbF proteins should be of ca. M_r 9,000 and 62,000, respectively. Since all PTS-dependent specific permeases are ca. M_r 66,000 (24, 25), it is possible that the *arbF*-encoded product is the EII specific permease. However, estimation of DNA fragment and protein sizes by electrophoresis can be misleading, and these predictions should be taken as tentative.

The pattern of *lacZ* fusions expression showed that both *arbB* and *arbF* genes are inducible and that β-glucoside-mediated induction requires ArbF, at least, and possibly ArbG. In contrast, *arbG* expression was not inducible and appeared to decrease in the presence of multiple copies of the *arb* genes. This would support the idea that there are two promoters that direct the expression of *arbG* and *arbF-arbB* genes, respectively. However, this seems at odds with the polar effect exerted by insertions in *arbG* upon *arbF* and *arbB* genes. As a working hypothesis, one can invoke a situation in which the *arbG* gene would be constitutively expressed for producing a protein activating the expression of downstream *arbF* and *arbB* genes.

When the *E. chrysanthemi arb* and *E. coli bgl* systems were compared in terms of substrate specificity, we observed that both mediate arbutin, salicin, and esculin fermentation, although the latter could not be used as a sole carbon source by either *E. coli* or *E. chrysanthemi*. Also, the genetic organization of both systems appeared to be the same; the permease and the phospho-β-glucosidase functions were encoded by the second and third genes, respectively (15, 29). In contrast, our results suggest that several differences could exist between the *E. coli bglG* antiterminator (1, 28) and the *arbG* gene: (i) fusion of both genes to a *lacZ* gene indicated that the basal level of *arbG* expression is much higher than that of *bglG*, (15), (ii) *arbG* expression is not substrate inducible, and (iii) a *bglG* mutation could not be compensated for by multiple copies of the *arbG* gene (pBH344) or of the *arbG-arbF* pair of genes (pBH334). However, concerning points (i) and (ii), one must be careful, since expressions of *bglG* and of *arbG* were performed by using *lacZ* transcriptional and translational fusions, respectively. Our current analysis of the *arbG*, *arbF*, and *arbB* nucleotide sequences should help us to establish the relatedness of both systems.

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