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Identification of an Ancillary Protein, YabF, Required for Activity of the KefC Glutathione-Gated Potassium Efflux System in *Escherichia coli*

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A new subunit, YabF, for the KefC K^+ efflux system in *Escherichia coli* has been identified. The subunit is required for maximum activity of KefC. Deletion of *yabF* reduces KefC activity 10-fold, and supply of YabF in *trans* restores activity. IS*2* and IS*10*R insertions in *yabF* can be isolated as suppressors of KefC activity consequent upon the V427A and D264A KefC mutations.

Glutathione (GSH)-gated potassium efflux systems are found in a range of gram-negative bacteria (5, 6). These systems have been most extensively studied for *Escherichia coli*, where there are two systems, KefC and KefB, that are closely related in their sequence and regulation (6). The efflux systems are maintained in a closed state by GSH or by its nonsulfydryl analogue, ophthalmic acid (10, 17). KefC and KefB are activated by adducts formed by reaction of GSH with electrophilic compounds, such as *N*-ethylmaleimide (NEM), methylglyoxal, and chlorodinitrobenzene (10). The systems differ in their response to methylglyoxal, with only KefB being strongly activated by this electrophile (12). In addition to regulation by specific ligands, the proteins share many features with eukaryotic channels, and calculated rates of K^+ efflux are consistent with channel-like activity (6). Each efflux system was originally identified as the product of a single structural gene, *kefC* and *kefB*, for the KefC and KefB systems, respectively (5, 6). In this study, we report that each system has in addition a separate and specific ancillary protein that is required for full activity.

The *yabF* gene was discovered during the cloning of the *kefC* locus from *Klebsiella aerogenes*. Plasmid pASRB1 was derived from a plasmid carrying the *K. aerogenes folA* gene, which in *E. coli* lies immediately clockwise after the *kefC* gene (3). This plasmid was expected to carry the *K. aerogenes kefC* gene since it restored NEM-elicited K^+ efflux to *E. coli* strain MJF276 (KefB$^-$ KefC$^-$) (26) and it complements the *E. coli* KefCD264A mutation (9). The sequence of the 4.4-kb *Eco*RI-*Bam*HI insert on pASRB1 was determined on both strands (26) (EMBL submission AJ242913) and was found to carry the 3′ end of the *carB* gene, *kefC*, and the open reading frame (orf) *yabF* (Fig. 1). On the *E. coli* chromosome, *kefC* and *carB* lie 13.7 kb apart and are separated by a number of orfs (4, 23), but the only one conserved at this position between *Klebsiella* and *E. coli* is *yabF*. PCR analysis using *K. aerogenes* cells as the source of DNA and a forward and reverse primer specific for *carB* and *kefC*, respectively, yielded a product of 2.4 kb, the size predicted from the sequence of the insert in pARSB1 (Fig. 1a) (data not shown). Similarly, Southern blots of *K. aerogenes* DNA digested with *Eco*RI and *Bam*HI using probes specific to *carB* and *kefC* yielded identical 4-kb bands as predicted by the restriction map of pARSB1 (Fig. 1a) (26). Thus, in the *K. aerogenes* genome *carB* and *kefC* are separated only by the orf *yabF*. The significance of this observation was enhanced by the observation that the *yabF* orf overlaps that of *kefC* by 8 bases in both *K. aerogenes* and *E. coli*, which suggested that the two genes might be related by function (Fig. 1b). A similar orf, *yheR*, was found 5′ to *kefB* and overlaps the 5′ end of the *kefB* gene of *E. coli* by 1 bp (20).

The *yabF* orf would encode a soluble 20.2-kDa protein (176 amino acids in *E. coli*) that exhibits considerable similarity to quinone oxidoreductases and to proteins involved in drug sensitivity (MdaB) (7) (Table 1). *K. aerogenes* YabF is only 83% identical to the *E. coli* protein (Table 1), whereas the KefC sequence retains 88% identity to *E. coli* KefC (data not shown). During the course of this study, the *yabF* gene of Frag5, the parent strain used in our studies, was found to have three single-base changes from the reported K-12 sequence (P31577), which cause two amino acid changes (N79D and G123V) and a silent alteration (G89). The identical sequence was found in pkC11, which we created in the initial cloning of the *yabF-kefC* genes (19), and which derives from the Clarke-Carbon cosmid series (8). These base changes were previously noted as conflicts (P31577) with the original K-12 sequence, but from our data it is likely that these differences are common to many *E. coli* K-12 strains.

To investigate the function of the YabF and YheR proteins, deletion mutants were constructed that removed *yabF* and *yheR* and the adjacent *kefB* and *kefC* genes. Regions flanking the *yabF-kefC* and *yheR-kefB* genes were amplified by PCR and cloned into pHG165 (2, 24). A restriction site was created between the flanking regions during the PCR amplification, as described previously (25), and the kanamycin resistance cassette of pUC4K (Pharmacia) was then inserted at this restriction site. Integration of the cloned kanamycin cassette DNA, via the flanking regions, was accomplished by transformation of strain JC7623 (15) as described previously (25). Strains MJF362 (Frag5, Δ*yabF-kefC*) and MJF369 (MJF274, Δ*yheR-kefB*) were created by P1 transduction of Frag5 and MJF274, respectively, to Kan^r (Table 2). From these and related strains carrying either *kefB*::Tn*10* or *kefC*::Tn*10* (5), a series of strains was created that possessed different combinations of YabF, YheR, KefC, and KefB (Table 2). The strains exhibited no significant growth phenotype. Using these strains, NEM-elicited KefC activity was investigated using two plasmids: pkC952 (YabF$^-$ KefC$^+$) (19), which expresses KefC but does not carry

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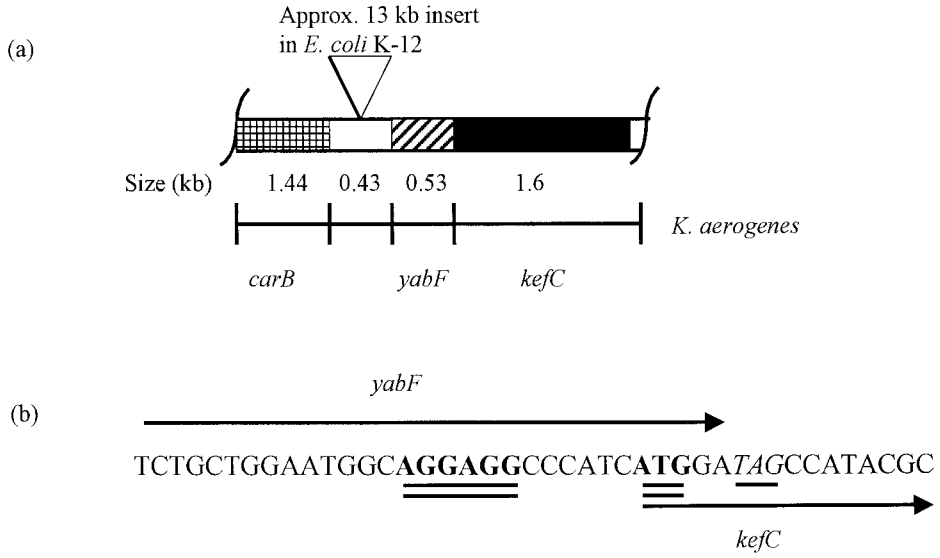

FIG. 1. Diagram showing the organization of the *carB* and *kefC* region in *K. aerogenes* and *E. coli* (a) and the overlap between *yabF* and *kefC* in *E. coli* (b). (a) The bar indicates the 4.2-kb fragment cloned and sequenced from pASRB1, showing the position of the 3′ end of *carB* (hatched), the *carB-yabF* intergenic region (open), *yabF* (diagonally striped), and *kefC* (filled). The inverted triangle above the block indicates the approximate position of the 13-kb insert found in the *E. coli* genome sequence (23). (b) DNA sequence of the *E. coli yabF-kefC* junction. The arrows indicate the position of the 3′ end of *yabF* (TAG stop codon underlined) and the 5′ end of *kefC* (putative Shine-Dalgarno sequence and start codon double underlined).

a complete *yabF* gene due to deletion of the first 389 bp of the *yabF* sequence at the 5′ end, and pkC11 ($YabF^+$ $KefC^+$) (9). The KefC activity derived from pkC952 was always less than that obtained with pkC11, as noted previously (9) (see below). Strain MJF276 ($YabF^+$ $KefC^-$ $YheR^+$ $KefB^-$), which retains a functional chromosomal *yabF* gene (12), exhibited high levels of KefC activity when transformed with either pkC952 or pkC11 (Fig. 2A). In contrast, MJF366 ($YabF^-$ $KefC^-$ $YheR^+$ $KefB^-$) exhibits only low activity when transformed with pkC592, while full efflux activity was seen with pkC11 (Fig. 2A). The residual K^+ efflux seen with MJF366/pkC952 was slightly greater than that seen with MJF276, which lacks KefB and KefC activity, and this suggests that the KefC system retains some activity in the absence of YabF (Fig. 2A). Identical data were obtained with strain MJF374 ($YheR^-$ $KefB^-$ $YabF^-$ $KefC^-$), which lacks both YabF and YheR, transformed with pkC952 and pkC11, and this suggests that the YheR protein could not substitute for YabF in activating KefC (data not shown). Thus, YabF is required for NEM-elicited KefC activity. Similar data obtained with plasmid pΔYheR ($YheR^-$ $KefB^+$) (20) support the conclusion that YheR is required for KefB activity (Fig. 2B). KefB activity was evident only when YheR either was present on the cloned fragment or was supplied from the chromosome (Fig. 2B). These data suggest that YabF and YheR proteins are required for KefC and KefB activity, respectively, and that this requirement can be met in *trans*.

Missense mutations in *kefC* that lead to enhanced spontaneous activity were previously isolated by their failure to grow on medium containing 0.1 mM K^+ ($K_{0.1}$ medium) (5, 11, 17).

TABLE 1. Sequences with greatest overall similarity to YabF

Gene sequence accession no.	Organism	Protein and/or function	BlastP score[a]	Similarity[b]		Length[d]
				%I	%S	
AJ242913	*K. aerogenes*	YabF; regulator of KefC	$1e^{-90}$	83	92	176/176
Z99117	*Bacillus subtilis*	YrkL; putative NAD(P)H oxidoreductase	$2e^{-30}$	39	56	170/174
Z93767	*Bacillus subtilis*	GS14; putative NAD(P)H oxidoreductase	$6e^{-29}$	38	54	163/175
U18997	*E. coli*	YheR; putative NAD(P)H oxidoreductase	$2e^{-26}$	40	55	163/184
Z99106	*Bacillus subtilis*	YdeQ; putative NAD(P)H oxidoreductase	$2e^{-26}$	38	54	164/197
J02888	*Homo sapiens*	Human quinone oxidoreductase (QR2)	$3e^{-13}$	33	48	149/231
P15559	*Homo sapiens*	Human quinone oxidoreductase (QR1)	$8e^{-12}$	34	56	103/274
T23934	*Streptomyces coelicolor*	Putative NAD(P)H oxidoreductase	$2e^{-7}$	37	58	65/246
U32829	*Haemophilus influenzae*	Putative NAD(P)H oxidoreductase	$4e^{-6}$	29	43	134/202
D90728	*E. coli*	Putative NAD(P)H oxidoreductase	$7e^{-6}$	31	46	105/196
C64084	*Haemophilus influenzae*	MdaB; modulator of drug activity[c]	$1e^{-5}$	23	37	178/208

[a] The BlastP (2.0.12) program (1) at the National Center for Biotechnology Information was used to compare the *E. coli* YabF protein sequence with available completed genomes. A number of related sequences have been found in the incomplete genome sequences also but have not been included in this analysis. None of the bacterial putative quinone oxidoreductases has been characterized.

[b] %I, percentage of identity; %S, percentage of similarity.

[c] A number of gene products that are related by sequence to the *E. coli* MdaB (modulator of drug resistance) (7) were found to be related to the YabF protein with similar BlastP scores but have been omitted for clarity. However, the *E. coli* MdaB protein exhibited a score of 0.005, which has a very low significance.

[d] First number, length of protein (amino acids) over which similarity is significant; second number, length of protein.

TABLE 2. Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Reference
Strains		
Frag5	F$^-$ Δ*kdpABC5 thi rha lacZ*	11
JC7623	F$^-$ *thr-1 leuB6 hisG4 argE3 thi-1 recB21 recC22 sbcB15 ara-14 tsx-33 supE44 galK2 rfbD1 zyl-5 mtl-1 rpsL31 lacY1* Δ(*gpt-proA*)*62 kdpK51*	15
MJF104	Frag5 *kefC104* (KefCD264A) *kefB*::Tn*10*	18
MJF104#29	MJF104 [IS*10*R 31 bp upstream of *yabF*]	This study
MJF104#31	MJF104 [IS*10*R 31 bp upstream of *yabF*]	This study
MJF116	Frag5 *kefC116* (KefCV427A) *kefB*::Tn*10*	18
MJF116#22	MJF116 [IS*2* 19 bp upstream of *yabF*]	This study
MJF116#5	MJF116 [*yabF*::IS*10*R; 434 bp 3′ to *yabF* ATG]	This study
MJF116#34	MJF116 [*yabF*::IS*10*R; 434 bp 3′ to *yabF* ATG]	This study
MJF116#52	MJF116 [*yabF*::IS*10*R; 436 bp 3′ to *yabF* ATG]	This study
MJF274	F$^-$ Δ*kdpABC5 thi rha lacI lacZ trkD1*	10
MJF276	MJF274 *kefB157 kefC*::Tn*10*	21
MJF335	MJF276 *gshA*::Tn*10*(Kan)	18
MJF362	Frag5 Δ[*yabF-kebfzztabftr;bC*]	26
MJF366	MJF274 *kefB*::Tn*10* Δ[*yabF-kefC*]	20
MJF369	MJF274 Δ[*yheR-kefB*]	20
MJF374	MJF276 Δ[*yabF-kefC*] Δ[*yheR-kefB*]	20
MJF526	MJF116#52 *gshA*::Tn*10*(Kan)	This study
MJF527	MJF116#22 *gshA*::Tn*10*(Kan)	This study
MJF528	MJF104#29 *gshA*::Tn*10*(Kan)	This study
MJF532	MJF116 *gshA*::Tn*10*(Kan)	This study
Plasmids		
pHG165	Cloning vector; a pBR322 copy number derivative of pUC8	24
pASRB1	pBR322 carrying *yabF* and *kefC* from *K. aerogenes* strain W70	3
pkC11	pHG165 carrying *yabF* and *kefC*	19
pkC952	pkC11 minus promoter region and first 389 bp of *yabF*	19
pCWyabF	*Xho*I-*Eco*RV fragment from pKC11; *yabF* and initial 471 bp of *kefC*	26
pKefB	pHG165 carrying *yheR* and *kefB*	20
pΔYheR	pKefB with promoter region and first 188 bp of *yheR* deleted	20
pGEXBG4	pGEX2TK carrying in-frame fusion of GSH *S*-transferase–YabF	14

We have previously shown that *kefC* null mutants are readily isolated as colonies that grow on $K_{0.1}$ medium (5, 11). These isolates grow normally on medium containing either 10 mM (K_{10}) or 115 mM (K_{115}) K^+, and thus buffers and media with these concentrations were used to allow normal growth and retention of the K^+ pool. Independent suppressor mutations were sought that restored growth on $K_{0.1}$ medium but which retained KefC activity as detected by NEM-elicited K^+ efflux. Strains carrying suppressor mutations were isolated from strains MJF104 and MJF116, which carry the KefCD264A and KefCV427A mutations, respectively (18). Overnight cultures of MJF104 or MJF116 (grown at 37°C in K_{120} minimal medium and glucose [0.2% (wt/vol)] as the carbon source) were washed sequentially in K_{10} buffer and K_0 buffer and then serially diluted in K_0 buffer. Dilutions were plated on control, high-potassium (K_{120}) medium, and selective, low-potassium ($K_{0.1}$) minimal medium (11). Colonies were visible after 48 h on the low-potassium plates, and a single suppressor colony was chosen from each original overnight culture. The frequency of isolation of suppressors of strains MJF116 and MJF104 was approximately 3×10^{-5} and 4×10^{-6}, respectively. Six mutant strains exhibited the unmodified *kefC* gene sequence of the mutant strains used for suppressor isolation (i.e., they retained either the V427A or the D264A mutation). However, PCR analysis of the *yabF* gene and its flanking regions revealed that the normal ~950-bp fragment found in the parent (Frag5), MJF104 (KefCD264A), and MJF116 (KefCV427A) was enlarged to approximately 2 to 3 kb. Sequence analysis on both strands revealed that this region of each suppressor strain contained an insertion of either IS*10*R or IS*2* (Table 2). The MJF104 suppressors MJF104#29 and MJF104#31 had IS*10*R insertions 5′ to the *yabF* gene approximately 31 bp 5′ to the translation start site. Although both IS*10*R insertions are in the same orientation, the strains were isolated from independent cultures. MJF116#22 was found to contain an IS*2* insertion 5′ to the *yabF* gene at approximately 19 bp 5′ to the translation start site, which may disrupt the promoter structure. Three MJF116 derivatives, MJF116#52, MJF116#5, and MJF116#34, carried an IS*10*R element inserted within the coding sequence of *yabF* (Table 2). The insertion in MJF116#5 and MJF116#34 was at the same position (434 bp into *yabF*), but the IS*10*R insertions lie in the opposite orientation (data not shown). Strain MJF116#52 carries an IS*10*R at bp 436 of *yabF* (data not shown). Thus, it is likely that the suppression in these strains arose either from inactivation of YabF or from reduced expression of *yabF* due to disruption of the promoter. Southern hybridization was performed on the suppressor strains and confirmed that the IS*10*R and IS*2* mutations arose by a single duplicative transposition from an insertion sequence (IS) located elsewhere in the genome (data not shown).

The activity of the KefC system was analyzed in more detail for strains MJF116#52 (KefCV427A, YabF::IS*10*R) and MJF116#22 (KefCV427A, IS*2* insertion 5′ to *yabF*). These strains differed in their response to the introduction of YabF in *trans*. Strain MJF116#22 showed only marginal increases in KefC activity when pCWYabF was introduced irrespective of whether GSH was present (Fig. 3A). Similar data were obtained with suppressors of MJF104 both of which had IS insertions 5′ to the *yabF* gene (data not shown). These data are

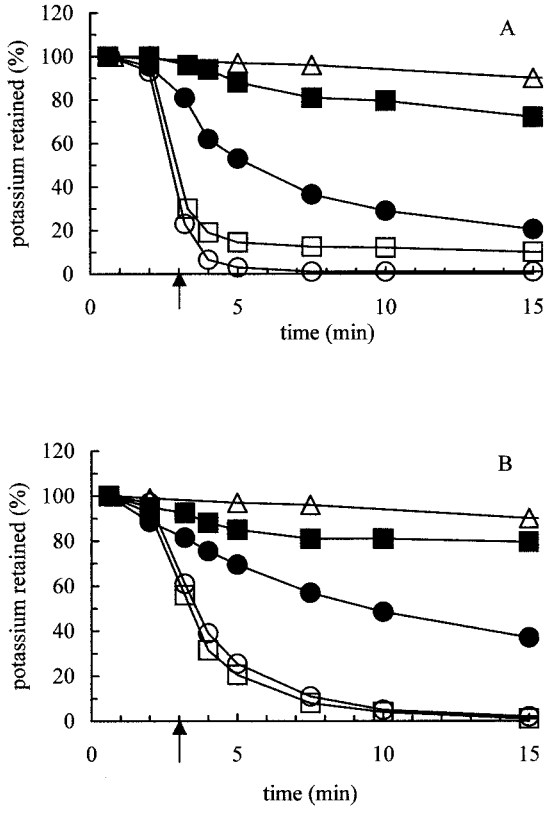

FIG. 2. YabF and YheR are required in *trans* for the activity of KefC and KefB. Potassium efflux was measured in mutant strains transformed with the appropriate plasmids to allow the contribution of YabF and YheR to be analyzed. Potassium efflux was measured according to methods in previous publications (21), and each experiment has been repeated at least three times. The data shown are representative. The arrow indicates the time of addition of 0.5 mM NEM. (A) KefC activity. Symbols: △, strain MJF276 ($YheR^+$ $KefB^-$ $YabF^+$ $KefC^-$); ●, MJF276/pkC952; ○, MJF276/pkC11; ■, MJF366/pkC952; □, MJF366/pkC11. (B) KefB activity. Symbols: △, strain MJF276; ●, MJF276/pΔYheR; ○, MJF276/pKefB; ■, MJF370/pΔYheR; □, MJF370/pKefB. Strain MJF276 expresses YabF and YheR, MJF366 expresses only YheR, and MJF370 expresses only YabF. Plasmids pkC952 ($YabF^-$ $KefC^+$), pkC11 ($YabF^+$ $KefC^+$), pΔYheR ($YheR^-$ $KefB^+$), and pKefB ($YheR^+$ $KefB^+$) are described in Table 2. Data obtained with MJF374 ($YabF^-$ $KefC^-$ $YheR^-$ $KefB^-$) transformed with the above plasmids were identical to the data obtained with either MJF366 (A) or MJF370 (B) (data not shown).

consistent with suppression arising from diminished expression of both YabF and KefC due to the IS insertion 5′ to the *yabF* gene. MJF116#52 (KefCV427A, YabF::IS*10*R) exhibited low rates of both spontaneous (i.e., in the absence of an added electrophile) and NEM-elicited efflux compared with the parent strain MJF116 (KefCV427A) (Fig. 3B). Efflux could be restored by expression of YabF in *trans* using plasmid pCW-YabF (Table 2), which carries the *E. coli yabF* gene (Fig. 3B), or by pGEXBG4, which expresses a GSH *S*-transferase–YabF protein fusion (data not shown) (14). Thus, the YabF protein expressed in *trans* can complement the YabF deficiency of this suppressor strain. GSH-deficient mutants exhibit high rates of spontaneous K^+ loss due to deregulation of KefC activity (10, 17, 18), and in most missense mutants of KefC there is synergy between the absence of GSH and the altered KefC protein leading to very rapid K^+ efflux (18). In contrast, K^+ efflux was negligible in the equivalent GSH-deficient derivative of strain MJF116#52 (KefCV427A, YabF::IS*10*R), and introduction of pCWYabF restored K^+ efflux (Fig. 3C). Thus, YabF is required for activity of KefC even in the absence of GSH.

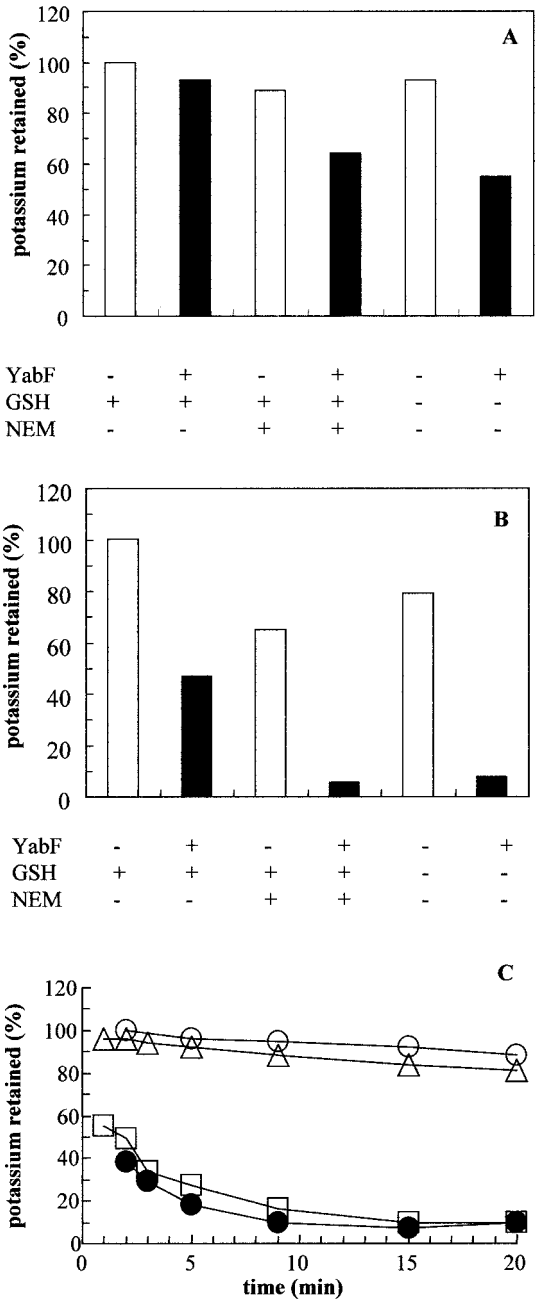

FIG. 3. Analysis of KefCV427A suppressor mutants. Potassium efflux was determined as described previously (10, 12). (A) Strains MJF116#22, (KefCV427A, IS*2* 5′ to *yabF*) and MJF527 (MJF116#22, $GshA^-$) were transformed with pCWYabF ($YabF^+$) to create strains that possess different combinations of YabF and GSH biosynthesis (Table 2). The strains were incubated in potassium-free medium (18), and either spontaneous K^+ efflux (no NEM) or NEM-elicited K^+ efflux (NEM added 3 min after resuspension in K^+-free medium) was measured. Open columns, $YabF^-$ strains; filled columns, $YabF^+$ strains. The percentage of K^+ retained 15 min after resuspension in K^+-free medium is shown. Low figures indicate high KefC activity. One hundred percent is set for MJF527 immediately after resuspension in K^+-free medium. (B) Details are as for panel A, substituting strains MJF116#52 and MJF526 (MJF116#52, $GshA^-$) for strains MJF116#22 and MJF527, respectively. Similar data were obtained with MJF116#5 and MJF116#34 and their $GshA^-$ derivatives (data not shown). (C) Potassium efflux from GSH-deficient strains: MJF335 (MJF276, $GshA^-$) (△), MJF532 (MJF116, $GshA^-$) (□), MJF526 (MJF116#52, $GshA^-$) (○), and MJF526/pCWYabF (●). Spontaneous K^+ efflux was measured as described previously (18), and the experiments have been repeated at least three times. The data shown are representative.

From these data, we conclude that YabF is an intrinsic component of the KefC potassium efflux system. A similar protein, YheR, was found for the activity of the KefB system. The ancillary subunits are specific to the individual efflux system since strains possessing YheR but lacking YabF did not display significant KefC activity (MJF366/pkC952 [Fig. 2A]) and vice versa (data not shown). YabF and YheR exhibit only 55% similarity, which may explain the apparent lack of cross activation of the efflux systems. The *yabF* and *yheR* orfs and the genes for the integral membrane components (*kefC* and *kefB*, respectively) of the efflux systems overlap in sequence and may form operons in *E. coli*. However, it is likely that the *kefC* gene can be expressed independently of *yabF*, since firstly pkC952 expresses KefC, albeit at an approximately 30-fold-lower rate than that of the normal *yabF-kefC* construct found in pkC11 (9, 12). Secondly, the *yabF*::IS suppressor mutants of the V427A and D264A mutants of KefC still retain expression of the KefC system, and complementation in *trans* by the cloned *yabF* gene restores full KefC activity (Fig. 3B and C). The overlap between the *yabF* and *kefC* genes may allow for translational coupling between the two orfs. There is strong secondary structure predicted for the *yabF-kefC* junction (and also for *yheR-kefB*), which places the ribosome binding site for the membrane protein in a stem-loop. This may be sufficient to explain the low level of expression from pkC952 (9, 12), since this plasmid does not carry the translation initiation signals for *yabF* but retains the 3′ sequence that is involved in potential stem-loop formation.

The regulation of KefC and KefB by GSH and GSH metabolites is well established (10, 17, 18). Regulation of K^+ efflux via KefC or KefB by GSH is unlikely to be directly mediated by the ancillary subunits since strains lacking YabF do not exhibit high rates of spontaneous potassium loss, which is the phenotype expected for loss of control by GSH (10, 17). Indeed, even in the absence of YabF and YheR, the KefC and KefB efflux systems retain both negative regulation by GSH and activation via GSH adducts (Fig. 2 and 3). The data presented here show that the newly identified subunit is required for maximum activity of the system and therefore provides an additional level of complexity of the efflux systems. The YabF protein shows strong sequence similarity to human quinone oxidoreductases QR1 and QR2 (13). YabF is a shorter protein than either QR1 or QR2, which arises from truncation of the N and C termini (data not shown). The greatest sequence conservation lies around two regions associated with flavin binding, but YabF retains only 4 of the 15 residues that are implicated in binding the flavin. Due to truncation of the C terminus, the YabF protein lacks the NAD(P)H binding site that is present in QR1 (13). It seems unlikely, therefore, that the YabF protein (and by inference YheR, which similarly lacks conservation of the essential residues) has quinone oxidoreductase activity. It seems probable that these proteins have evolved from their role as oxidoreductases to be modulators of KefC (and KefB) activity in a manner similar to that of β subunits of mammalian *Shaker* channels (16). Therefore, by analogy with the *Shaker* family of K^+ channels (16, 22), we suggest that the KefB and KefC efflux systems each comprise two structural components, KefC with YabF and KefB with YheR, both of which are required to give the functional characteristics of the efflux system. Given their unique role in the activity of KefC and KefB, we now propose that YabF and YheR should be termed KefF and KefG, respectively.

S.M., L.S.N., and C.M.W. contributed equally to the work.

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