Noncontiguous operon is a genetic organization for coordinating bacterial gene expression

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Bacterial genes are typically grouped into operons defined as clusters of adjacent genes encoding for proteins that fill related roles and are transcribed into a single polycistronic mRNA molecule. This simple organization provides an efficient mechanism to coordinate the expression of neighboring genes and is at the basis of gene regulation in bacteria. Here, we report the existence of a higher level of organization in operon structure that we named noncontiguous operon and consists in an operon containing a gene(s) that is transcribed in the opposite direction to the rest of the operon. This transcriptional architecture is exemplified by the genes menE-menC-MW1733-yrkD-MW1731 involved in menaquinone synthesis in the major human pathogen Staphylococcus aureus. We show that menE-menC-yrkD-MW1731 genes are transcribed as a single transcription unit, whereas the MW1733 gene, located between menC and yrkD, is transcribed in the opposite direction. This genomic organization generates overlapping transcripts whose expression is mutually regulated by transcriptional interference and RNase III processing at the overlapping region. In light of our results, the canonical view of operon structure should be revisited by including this operon arrangement in which cotranscription and overlapping transcription are combined to coordinate functionally related gene expression.

operon | antisense transcription | overlapping transcription | RNase III | menaquinone

The term operon was first proposed by Jacob and Monod (1) as a functional genomic DNA unit containing a group of genes that are transcribed together under the control of a single promoter. This concept served to explain a revolutionary model of bacterial gene regulation, in which expression of a cluster of genes was negatively controlled by a repressor acting at a single operator that coordinated the process. Over time, it has been shown that operon gene regulation is much more complex than originally expected, with operons that can be positively and/or negatively regulated at different levels other than transcription initiation (2–4). The overall simplicity of operon organization for coordinating gene expression explains why a substantial fraction of functionally related bacterial genes are organized into operons (5, 6).

The list of operons in a specific genome can be predicted with reasonable accuracy based on features such as the distance between each adjacent gene, the likelihood of a pair of genes to be neighbors in a group of reference genomes, and the phylogenetic distance (7–9). However, these features are predisposed by the classical operon concept, and, consequently, the identification of variations in genetic organization inside operon structure through bioinformatic predictions has been very limited. The development of RNA deep sequencing technologies is helping to elucidate “operons” in their full complexities by precisely defining the beginning and the end of mRNA molecules and revealing the changes in the structure of statically predicted operons under different experimental conditions (4, 10–17). An unexpected finding unraveled by the precise determination of transcript boundaries is that very often convergent operons overlap at their 3′ end (tail to tail) and divergent operons overlap at their 5′ end (head to head). In these situations, the mRNA contributes to the expression of operon genes, and at the same time, a region of the mRNA acts as an antisense transcript, affecting the expression of the contiguous operon. The simultaneous sense and antisense functions for transcript boundaries were reported under the term excludon in Listeria monocytogenes (18). This genomic organization allows the establishment of a regulatory relationship that results in the “exclusive” expression of only one of both coding regions. The mechanisms for excludon-mediated regulation are multifaceted and it can include transcription interference, transcription attenuation, degradation of the double-stranded overlapping RNAs, or stabilization of the RNAs after cleavage (19, 20).

In a previous work, and through a genome-wide transcriptome profiling of the pathogen Staphylococcus aureus (21), we identified several examples of groups of genes that were apparently transcribed together despite that they were separated by gene(s) transcribed in the opposite direction. This transcriptional organization is an extreme example of an excludon, since the mRNA encoded on the opposite strand of DNA to the operon would serve as a canonical mRNA that encodes for a protein while acting as an antisense RNA, base-pairing all along its length with an internal untranslated region of the polycistronic mRNA. Here, we report the existence of this transcriptional organization in an operon involved in the synthesis of menaquinone in S. aureus. Our results demonstrate that the expression of both overlapping transcripts is mutually regulated by transcriptional interference and endoribonuclease-mediated digestion. The existence of this genetic arrangement,

Significance

In bacteria, functionally related genes are often cotranscribed in a single mRNA molecule under the same upstream promoter, forming a polycistronic operon unit. With this strategy, bacteria guarantee that production of all proteins related to a specific cellular process is simultaneously switched on or off. Here, we report the identification of a transcriptional organization consisting in operons that contain a gene(s) that is transcribed in the opposite direction to the rest of the genes of the operon. As a consequence, the resulting mRNA is fully complementary to the operon transcript. This genetic arrangement leads to mutual regulation of the overlapping transcripts expression and, thus, provides an additional strategy for coordinating the expression of functionally related genes within an operon.


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which we named noncontiguous operon, confirms overlapping transcription as a specific mechanism for regulating gene expression within an operon. In addition, it underlines the relevance of reviewing operon structures in bacterial genomes to identify all protein partners whose expression is coordinated in a particular cellular process.

Results

Identification of "Noncontiguous Operons" in the S. aureus Genome.

We screened genome-wide the transcriptome data obtained from the clinical isolates S. aureus 15981 (21) and S. aureus MW2 to identify genes cotranscribed together despite being separated by a gene transcribed in the opposite direction. We found six examples that fit the predicted model (Fig. 1 and SI Appendix, Figs. S1 and S2). RNA sequencing data of published results from different laboratories (rnamaps.unavarra.es/) confirmed the existence of identical transcriptional organizations in five other genetically unrelated S. aureus strains (Fig. 1 and SI Appendix, Figs. S1 and S2). The function of most of the proteins encoded by such operons is unknown. CoaD, which is part of the CoA biosynthesis pathway, MenADB and MenEC, required for menaquinone synthesis, and MoaABCED, required for molybdate transport, are among the proteins with known functions.

To explore the significance of the transcriptional organization, we chose the region comprising menE, menC, MW1733, ytkD, and MW1731 genes based on the size of the transcripts and the relevance of menaquinone synthesis during S. aureus infections (22) (Fig. 1). menE-menC and ytkD-MW1731 genes are listed as two independent operons in the prokaryotic operons database (csbl.bmb.uga.edu/DOOR/) (23). However, transcriptome data indicated that both operons are transcribed as a single transcriptional unit (Fig. 1). These results agreed with published results obtained by mapping of transcriptional start sites (TSS) by differential RNA-seq that revealed a unique TSS upstream the menE gene (24) (Fig. 1). To experimentally confirm the transcriptome results and because the environmental conditions controlling the expression of menE remain unknown, we first generated two derivatives of the wild-type strain in which the promoter region upstream the menE gene was deleted (Δmen strain) or replaced by the constitutive blaZ promoter (PblaZ-men strain) (SI Appendix, Fig. S3). For each of these strains, we generated derivatives in which the chromosomal copy of either menC or MW1731 genes was tagged with the 3xFLAG sequence (SI Appendix, Fig. S3) and then examined MenC and MW1731 protein levels by Western blotting. Consistent with the transcriptome results, the Δmen mutation correlated with inhibition of both MenC and MW1731 proteins expression. Note that the Δmen deletion did not completely abolish protein production. On the contrary, the strains containing the constitutive blaZ promoter produced considerably higher levels of MenC and MW1731 compared with the wild-type strain (Fig. 2A). Thus, these findings suggested that expression of ytkD-MW1731 depends on the promoter region upstream the menE gene.

To further validate the cotranscription of menE-menC-ytkD-MW1731 genes, we performed Northern blot analysis using strand-specific riboprobes corresponding to menE-menC (probe A) and ytkD-MW1731 (probe B) coding regions with total RNA from exponentially growing cells of the wild-type strain, and its two isogenic derivatives, Δmen and PblaZ-men. Results showed that the mRNA expression levels are very low in the wild-type strain, because neither probe was able to detect the mRNA (Fig. 2B). In contrast, both probe A and B hybridizations with PblaZ-men RNA revealed an increased accumulation of a fuzzy band of ∼4 kb that was compatible with cotranscription of menE-menC with the downstream genes ytkD-MW1731 (Fig. 2B). Note that probe B also clearly detects an additional processing band (∼1.2 kb). Together, these results strongly suggest that menE-menC and ytkD-MW1731 expression depends on the promoter located upstream menE, which is consistent with transcriptome data indicating that menE-menC-ytkD-MW1731 genes comprise a single transcriptional unit.

Transcriptome data also indicated that the MW1733 gene (258 bp long) is transcribed at high levels with a short 5' UTR of 26 nucleotides and a 3' UTR of 60 nucleotides that overlaps the 3' end of the menC coding sequence. To confirm transcriptome data, we generated two additional strains in which 27 nucleotides of the promoter region upstream the MW1733 gene were deleted (∆PMW1733 strain) or replaced by the constitutive blaZ promoter (PblaZ-MW1733 strain) (SI Appendix, Fig. S3). For each of these strains, we generated a derivative in which the chromosomal copy of the MW1733 gene was tagged with the 3xFLAG sequence and RNA-seq data were obtained from 15981 (21), MW2 (this study), UAMS-1 (41), HG001 (42), WCH-5K2 (43), Homeland (24), and USA300-P23 (44).

Fig. 1. Analysis of the noncontiguous operon architecture. JBrowser software images showing RNA-seq or TSS-seq mapped reads distribution in the region comprising menE-menC-MW1733-ytkD-MW1731 genes of seven unrelated S. aureus strains. The scale (log2 or ×103) indicates the number of mapped reads per nucleotide position. A schematic representation of the structure under study is shown in the middle of the scheme. ORFs are represented as orange arrows for the genes that constitute the menE-menC-ytkD-MW1731 operon and as a blue arrow for the MW1733 gene. Promoters are shown as green triangles and transcriptional terminators as red rectangles. The transcript generated from the menE-menC-ytkD-MW1731 operon is represented as a dashed orange arrow, while the transcript generated from MW1733 is presented as a dashed blue arrow. The top line denotes the position in base pairs of the S. aureus MW2 genome. All genetic information about the start and the end of transcription was obtained from a previous study (21). RNA-seq data were obtained from 15981 (21), MW2 (this study), UAMS-1 (41), HG001 (42), WCH-5K2 (43), Homeland (24), and USA300-P23 (44).
The Expression of the menE-menC-ytkD-MW1731 Operon and the MW1733 Gene Is Reciprocally Regulated. To determine whether transcriptional levels of menE-menC-ytkD-MW1731 have an effect on the amount of MW1733 mRNA, we compared Northern blot the transcript levels of MW1733 in the wild-type, Δmen and PblaZ-men strains using probe C to detect MW1733 mRNA. Results showed that MW1733 transcript levels slightly increased when transcription of the operon was inhibited and, on the other hand, markedly decreased in PblaZ-men strain, which is under the presence of an excess of the overlapping tetracistronic transcript (Fig. 3A). To confirm the regulation of MW1733 expression at a protein level, we constructed derivatives of Δmen and PblaZ-men containing the chromosomal copy of the MW1733 gene tagged with a 3xFLAG epitope at the C terminus (SI Appendix, Fig. S4). Consistent with Northern blot results, MW1733 protein levels significantly decreased in PblaZ-men compared with those in the wild-type strain (Fig. 3A).

Next, we investigated the possibility of a reciprocal effect of MW1733 mRNA levels on the expression of the tetracistronic operon. To do so, we first analyzed by Northern blot, and with the use of probe A, menE-menC-ytkD-MW1731 mRNA levels in the wild-type, ΔPMW1733, and PblaZ-MW1733 strains. In agreement with the low level of expression of the tetracistronic mRNA in the wild-type strain (Fig. 2B), we could not find a significant difference in menE-menC-ytkD-MW1731 mRNA levels between strains when probe A was used (Fig. 3B). Thus, we repeated the Northern blot assay with the use of probe B, specific for ytkD-MW1731. Again, the ytkD-MW1731 transcript was undetectable in the wild-type and ΔPMW1733 strains. However, when MW1733 was overexpressed, a specific processing transcript was detected. The size of the discrete band (∼1.5 kb) is consistent with a transcript including ytkD-MW1731 that might be obtained upon processing of the menE-menC-ytkD-MW1731 mRNA (Fig. 3C). Next, we constructed derivatives of ΔPMW1733 and PblaZ-MW1733 harboring a chromosomal copy of either menC or MW1731 tagged with the 3xFLAG epitope in the carboxyl-terminal domain (SI Appendix, Fig. S4). Notably, constitutive expression of MW1733 caused a clear reduction in the levels of the MenC protein (Fig. 3B) and a significant accumulation of MW1731 protein levels in PblaZ-MW1733 compared with the wild-type strain (Fig. 3C).

Collectively, these results support the notion that in the noncontiguous operon, transcriptional units generated from opposite

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**Fig. 2.** Experimental evidence showing that the region comprising menE-menC-MW1733-ytkD-MW1731 genes exhibits an architecture characteristic of a noncontiguous operon. (A) Western blots showing MenC, MW1731, or MW1733 protein levels in the WT and the following derivative strains: Δmen, PblaZ-men, ΔPMW1733, and PblaZ-MW1733. The 3xFLAG-tagged proteins were detected with commercial anti-3xFLAG antibodies. Coomassie stained or stain-free gel portions are shown as a loading control. (B) Northern blot analysis of RNA harvested from the strains described in A. Blots were probed with specific riboprobes for menEC, ytkD-MW1731, and MW1733 regions. The positions of RNA standards are indicated. Lower shows 16S and 23S ribosome bands stained with ethidium bromide as loading control. The strains used in this figure are depicted in SI Appendix, Fig. S3.

**Fig. 3.** Mutual regulation of overlapping transcripts expression within the noncontiguous operon. (A, Upper) Northern blot showing MW1733 mRNA levels in the WT, Δmen, and PblaZ-men strains. A specific riboprobe (probe C) for MW1733 was used; Western blot showing MW1733 protein levels in the same strains producing a 3xFLAG-tagged MW1733 protein (A, Lower). (B, Upper) Northern blot showing menE-menC mRNA levels in the WT, ΔPMW1733, and PblaZ-MW1733 strains. A specific riboprobe (probe A) for menEC was used; Western blot showing MenC protein levels in the same strains producing a 3xFLAG-tagged MenC protein (B, Lower). (C, Upper) Northern blot showing ytkD-MW1731 mRNA levels in the strains described in B. A specific riboprobe (probe B) for ytkD-MW1731 was used; Western blot showing MW1731 protein levels in the same strains producing a 3xFLAG-tagged MW1731 protein (C, Lower). The positions of RNA standards are indicated and 16S and 235 ribosome bands stained with ethidium bromide are shown as loading controls. The 3xFLAG-tagged proteins were detected with commercial anti-3xFLAG antibodies. Coomassie stained or stain-free gel portions are shown as a loading control. The strains used in this figure are depicted in SI Appendix, Fig. S4.
strands regulate each other's expression. Thus, in the noncontiguous operon under study, an increase in tetracistronic operon transcription negatively regulates the expression of the interspersed MW1733 gene. Reciprocally, an increase in MW1733 mRNA discoordinates expression within the overlapped operon, by strongly elevating ytkD-MW1731 mRNA levels while reducing menE-menC expression.

Analysis of the Mechanisms Underlying the Regulation of Noncontiguous Operons Expression. RNase III endoribonuclease is responsible for processing overlapping sense/antisense transcripts genomewide in bacteria (21, 25, 26). Thus, we examined the importance of RNase III activity in the reduction of MW1733 transcript levels when an excess of menE-menC-ytkD-MW1731 is transcribed. Deletion of RNase III both in the wild-type strain and in the strain overproducing the tetracistronic operon (PblaZ-men) (SI Appendix, Fig. S5) caused a slight increase in the amount of MW1733 mRNA (Fig. 4A). Consequently, MW1733 protein levels only moderately increased in rnc mutants compared with those in the respective RNase III producing strains (Fig. 4A). However, we studied the involvement of RNase III in menE-menC-ytkD-MW1731 mRNA processing when MW1733 is overexpressed. A Northern blot, using probe A, with RNA from cells of the wild type, PblaZ-MW1733, and their corresponding rnc mutants showed no significant differences between strains, given the low detectability of menE-menC-ytkD-MW1731 mRNA (Fig. 4B). Second, we carried out a similar Northern blot, but with the use of probe B, specific to detect ytkD-MW1731 mRNA. Results revealed that processing of the tetracistronic mRNA when an excess of MW1733 is transcribed still occurred in the absence of RNase III. However, in this case, the processing pattern of the operon changed, leading to a significant decrease in the amount of the discrete 1.5-1kb transcript and to the appearance of two additional larger mRNA fragments (Fig. 4C). Accordingly, MW1731 protein levels decreased in rnc mutants of the wild-type and PblaZ-MW1731 strains compared with those in their respective RNase III producing strains (Fig. 4C and SI Appendix, Fig. S5). Overall, these results indicated that RNase III explains, only to a certain extent, the MW1733-mediated cleavage of menE-menC-ytkD-MW1731 mRNA, suggesting that additional ribonuclease(s) might also be responsible for this processing.

Besides processing by RNase III, another possible explanation for the reciprocal regulation of overlapping transcripts described above might be transcriptional interference (27), defined as the suppressive influence that convergent RNA synthesis machinery from one DNA strand causes in cis on the transcription of the neighboring gene. Thus, we next sought to determine whether the observed antisense regulation of menE-menC-ytkD-MW1731 over MW1733 occurred when the MW1733 gene was expressed in another location of the chromosome. To do so, we inserted a 3xFLAG-tagged MW1733 gene under its own promoter next to the innocuous attB site of the lipase gene in both ΔPMW1733 and ΔPMW1731 PblaZ-men genetic backgrounds (SI Appendix, Fig. S6). Importantly, and contrary to what happens when MW1733 is located in its natural location, Northern blot analysis of MW1733 transcript levels showed that these were only slightly reduced in the presence of an excess of menE-menC-ytkD-MW1731 mRNA when the MW1733 gene was placed and expressed in trans (Fig. 4D). Note that there is a marked difference in the size and abundance of MW1733 mRNA when it is ectopically expressed from the attB chromosomal location. Consistent with Northern blot results, Western blot analysis showed that MW1733 protein levels were unaffected in the ΔPMW1733 PblaZ-men MW1733 trans strain compared with those in the wild-type strain (Fig. 4D). These results indicated that menE-menC-ytkD-MW1731-mediated suppressive influence on MW1733 expression requires cis localization of both transcripts. Lastly, to reinforce these results, we overexpressed a 3xFLAG-tagged MW1733 gene ectopically from a plasmid in the wild-type strain harboring a chromosomal copy of either menC or MW1731 tagged with the 3xFLAG epitope (SI Appendix, Fig. S6) and analyzed MenC and MW1731 levels by Western blot.

Fig. 4. RNase III processing at the overlapping region and transcriptional interference are involved in reciprocal regulation of the overlapping transcripts generated from the noncontiguous operon. (A, Upper) Northern blot showing MW1733 mRNA levels in the WT, ΔmenMW1733, Δrnc, PblaZ-men, and PblaZ-men Δrnc. A specific riboprobe (probe C) for MW1733 was used; Western blot showing MW1733 protein levels in the same strains producing a 3xFLAG-tagged MW1733 protein (A, Lower). (B, Upper) Northern blot showing menE-menC mRNA levels in the WT, ΔmenMW1733, Δrnc, PblaZ-MW1733, and PblaZ-WMW1733 Δmen. A specific riboprobe (probe A) for menE-menC was used; Western blot showing MenC protein levels in the same strains producing a 3xFLAG-tagged MenC protein (B, Lower). (C, Upper) Northern blot showing ytkD-MW1731 mRNA levels in the strains described in B. A specific riboprobe (probe B) for ytkD-MW1731 was used; (C, Lower) Western blot showing MW1731 protein levels in the same strains producing a 3xFLAG-tagged MW1731 protein. (D, Upper) Northern blot showing MW1733 mRNA levels in the WT, PblaZ-men, ΔPMW1733, ΔPMW1733 MW1733 trans and ΔPMW1731 PblaZ-men MW1733 trans. A specific riboprobe (probe C) for MW1733 was used; Western blot showing MW1733 protein levels in the same strains producing a 3xFLAG-tagged MW1733 protein (D, Lower). (E) Western blot showing MenC (Upper) and MW1733 (Lower) protein levels in the WT, WT pCN40, PblaZ-MW1733, and WT pCN40::MW1733-3xFLAG. Strains contained a chromosomal copy of either menC or MW1731 tagged with the 3xFLAG epitope. The positions of RNA standards are indicated, and 18S and 23S ribosome bands stained with ethidium bromide are shown as loading controls. The 3xFLAG-tagged proteins were detected with commercial anti-3xFLAG antibodies. Coomassie stained or stain-free gel portions are shown as a loading control. The strains used in this figure are depicted in SI Appendix, Fig. S5 for A–C and in SI Appendix, Fig. S6 for D and E.
Overexpression of MW1733 in trans did not have any impact on MenC or MW1731 production, showing that MW1733 effect in discoordinating menE-menC-yikD-MW1731 operon expression also requires cis localization of both transcripts (Fig. 4E).

Overall, the above results indicate the existence of a transcriptional interference mechanism of gene regulation between the machinery that synthesizes the noncontiguous operon mRNA and the one synthesizing the mRNA of the interspersed gene.

High Transcriptional Levels of the MW1732 Gene Can Lead to the Appearance of Small Colony Variants. The experiments shown above demonstrated that overexpression of MW1733 mRNA leads to reduced MenC protein levels (Fig. 3B). In S. aureus, the inhibition of the synthesis of menaquinone has been associated with a slowed growth phenotype, known as small colony variants (SCVs) (28). SCVs are frequently isolated from clinical samples obtained from patients experiencing chronic infections by S. aureus. We observed that the Δmen strain constructed in this work, which still shows some residual production of the MenC protein (Fig. 2A), produces colonies whose size are smaller than the ones corresponding to the wild-type strain although they are not as small as the SCVs generated by deletion of menE-menC genes (ΔmenEC) (SI Appendix, Fig. S7A). Therefore, we wondered whether constitutive expression of MW1733 might be followed by the appearance of SCVs phenotypic hallmarks. To test this hypothesis, the promoter of MW1733 was replaced by the constitutive blaZ promoter in Δmen strain (SI Appendix, Fig. S8). The resulting strain produced colonies significantly smaller than the Δmen strain and exhibited several characteristics associated to S. aureus SCVs such as decreased pigmentation and increased resistance to aminoglycosides (tobramycin, streptomycin, gentamycin, and amikacin) than the wild-type strain (29) (SI Appendix, Fig. S7 B and C). These results suggest that overexpression of MW1733 suppresses the expression of its convergent menE-menC genes, which, in turn, leads to suppressed menaquinone synthesis and the appearance of a SCV phenotype.

To confirm that appearance of SCVs by MW1733 overexpression in Δmen strain exclusively happened when MW1733 and menE-menC-yikD-MW1731 mRNAs were expressed in cis, we overexpressed the MW1733 gene ectopically from a plasmid in Δmen strain and analyzed colony size on TSA plates. The resulting strain, Δmen pCm40::MW1733 (SI Appendix, Figs. S7A and S8), showed the same phenotype as the Δmen strain. Thus, we conclude that this noncontiguous operon transcriptional organization constitutes an effective mechanism for regulating gene expression and ultimately for controlling cell growth.

Discussion

The novelty introduced by the noncontiguous operon concept is that genes within an operon can be interpersed with genes divergently transcribed and that, consequently, they do not necessarily need to be contiguous in the genome. This transcriptional arrangement does not fit within the classical operon paradigm, explaining why it has passed previously unnoticed. It is important to note that in all of the examples of noncontiguous operons in the S. aureus genome, coding sequences of the operon never overlap the coding region of the interspersed gene. Thus, it appears that the noncontiguous operon transcriptional architecture may be a result of evolutionary pressure to minimize genome size and provide an additional strategy for coupling the expression of functionally related polypeptides. Our results provide evidence of two mechanisms by which the noncontiguous operon arrangement can coordinate gene expression. The first mechanism is related with the generation of double-stranded templates between complementary overlapping RNAs that can modify mRNA stability or translation (30, 31). We showed that RNase III digestion of the mRNA duplexes is partially responsible for both the repression of MW1733 expression and also for the cleavage of the tetracistronic mRNA into two independent transcripts. The resulting two halves might be translated into proteins at a similar or different rate than before the cleavage. Our results indicate that transcriptional induction of the MW1733 gene leads on one hand to a reduction in MenE protein levels, and on the other, to the stabilization of the yikD-MW1733 half and, thus, to the accumulation of higher levels of MW1731 protein compared with the wild-type strain. Specific RNase III cleavage at intercistronic regions with alternative outcomes for the resulting mRNAs has been previously reported in Escherichia coli (32). Opdyke et al. showed that binding of the cis noncoding RNA gadY to the intercistronic region of gadXW mRNA resulted in RNase III cleavage and monocistronic transcripts accumulation, probably due to increased stability of single transcripts. Similarly, binding of a cis-encoded noncoding RNA to the cII-O mRNA of λ phage has been shown to be responsible for an RNase III processing event that is followed by degradation of the upstream cII fragment while the downstream O mRNA remains stable. Because the sRNA partially overlaps the cII coding sequence at its 3′ end, it was concluded that degradation of the cII transcript is due to RNase III processing occurring at that region (33). Regarding the mechanisms underlying the stabilization process, it is possible that cleavage might alter the secondary structure of the transcripts so that they are less susceptible to degradation. RNase III is not the only endonuclease involved in MW1733-dependent processing of the menE-menC-yikD-MW1731 operon because discrete RNA fragments from the tetracistronic operon are still detected in the absence of RNase III when MW1733 is overexpressed. An important direction for future studies should be to identify such additional endoribonucleases(s).

The second mechanism that contributes to coordinating mRNA expression within the noncontiguous operon is transcriptional interference. Because the distance between promoters of the tetracistronic operon and the MW1733 gene is longer than 200 nucleotides, the most obvious explanation for transcriptional interference is the collision between the RNA synthesis machinery from one DNA strand with the transcription machinery from the other strand (34, 35). A major finding consistent with the existence of transcriptional interference is that tetracistronic operon overexpression did not cause any effect on MW1733 mRNA levels when this was expressed in trans from a separate genomic location. Similarly, the expression of menE and MW1731 was unaffected when MW1733 was overexpressed in trans with MW1733. Therefore, the base-pairing between complementary transcripts can occur regardless of whether they are expressed in cis or trans, and therefore, digestion of overlapping transcripts by RNase III and additional endoribonucleases should take place when MW1733 is produced in trans. Thus, we currently do not understand why MW1733 overexpression in trans does not affect menC and MW1733 expression. One possibility is that pairing and processing of the overlapping transcripts is less efficient when both complementary transcripts are produced from separate genomic locations.

What are the benefits of the noncontiguous operon organization compared with regular operons? The exact functions of overlapping transcription are still a matter of debate, and several authors defend that overlapping transcription are mainly the product of transcriptional noise, arising at spurious promoters throughout the genome (36). The existence and maintenance of noncontiguous operon transcriptional architecture is strong evidence that overlapping transcription represents a specific strategy for gene regulation. We can imagine a number of ways the noncontiguous operon may create higher-level organizational features that are adaptive compared with a regular operon. First, it enables a disordinated expression within the genes of the operon upstream and downstream the overlapping gene, diminishing gene expression noise and ensuring a more precise stoichiometry. Second, it allows endoribonuclease-dependent removal of transcripts that escape the regular transcription repression process. Third, it allows down-regulation (exclusion) of the overlapping gene...
expression by transcript-independent transcriptional interference. Finally, it saves space and decreases the genetic load associated with selecting for a regulatory given motif. All these theoretical benefits require future studies to fully explore the fitness advantages that this transcriptional organization provides to bacteria.

The *menE-menC-MW1733-ytk-MW1731* genetic arrangement is conserved across the *Staphylococcus* genus, a fact suggesting high functional relevance (*SI Appendix, Fig. S9*). We have found an example of the regulatory possibilities of this transcriptional arrangement in the emergence of an SCVs phenotype associated to menaquinone synthesis deficiency in *S. aureus* (37). Many efforts have been made to identify auxotrophic mutations that result in the appearance of SCVs (28, 38). However, when examining *S. aureus* clinical and tissue-cultured induced SCVs, only around 20% can be assigned to a defined auxotrophy, implying that other pathways underlying SCVs formation probably exist (37). Here, we have seen that an increase in the transcription of *MW1733* can account for the induction of SCVs under low polyolic operon transcription levels without the need to generate a mutation. The generation of SCVs through this mechanism has the advantage of producing variants able to rapidly switch and revert to the fast-growing wild-type phenotype at the earliest opportunity to generate and infection, without the fitness cost associated with the generation of independent mutations and revertant mutations. In this way, the formation and stability of SCVs would be modulated by environmental conditions affecting transcriptional levels of both the *menE-menCytk-MW1731* operon and the *MW1733* gene and also by factors affecting the binding between overlapping transcripts and the RNase III processing rate. Further work is needed to identify environmental stimuli able to trigger SCVs through this mechanism.

Overall, our results add a further degree of complexity to the initial model of operon gene regulation described by F. Jacob and J. Monod and highlight the functional relevance of overlapping transcription as a mechanism to coordinate the expression levels of bacterial neighboring genes.

**Materials and Methods**

*S. aureus* strain 15981 was used as the genetic background for all genetic manipulations. A summary of strains used is provided in *SI Appendix, Table S1*. Mutant strains, 3xFLAG-tagged strains, strains harboring the PbaIA promoter instead of native promoters, and contaminants containing a 3xFLAG-tagged MW1733 gene under its own promoter next to the attB site of the lipase gene were generated via allelic replacement using the pMAD vector (39) as described (40). For inactivation of *rnc* (RNaseII encoding gene), the previously described pMAD Δ*rnc::catR* plasmid (21) was used. Detailed materials and methods are described in *SI Appendix, Materials and Methods*.

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