

accounted for by loss of function of various kinesin superfamily members (20). Control embryos injected with buffer alone did not exhibit any of these phenotypes (Fig. 3, A and C); the majority of them survived until the gastrula stage (Fig. 3E), when they were fixed and stained. Embryos injected in the post-cellularization stage developed normally, confirming that AS-2 was unable to cross cell membranes.

Our results demonstrate that AS-2 specifically inhibits kinesin activity by interfering with MT binding. This mechanism is unlike that of any known kinesin (or other motor) inhibitor and may be generated by AS-2 emulating tubulin binding to a portion of the MT-binding site of kinesin. AS-2 is also a potent toxin, which when delivered intracellularly may ablate several, if not all, aspects of kinesin-superfamily-mediated transport. AS-2 and its derivatives have many potential applications. When made membrane permeant, AS-2 and its derivatives would be likely to be efficient antimetabolic or antitransport drugs for studying kinesin functions or might be therapeutic agents. The existence of a kinesin atomic structure may allow the rational design of molecules based on AS-2 and the development of specific inhibitors for kinesin families and subfamilies, thus leading to precise chemical intervention. Finally, the ability of AS-2 and its derivatives to mimic the activity of the MT may allow modification of surfaces or other substrates with AS-2 to create artificial kinesin tracks.

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- Subsequent binding and kinetic measurements were performed on a bacterially expressed *Drosophila* kinesin heavy-chain fragment (K5-351) containing amino acids 5 through 351 and a hexahistidine tag at the COOH-terminus. Soluble protein was purified from isopropyl- β -D-thiogalactopyranoside-induced bacterial cells by a single round of affinity chromatography on Ni-NTA-agarose (Qiagen), concentrated by microfiltration, and frozen in aliquots in liquid nitrogen.
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- Embryos were collected every 20 min and dechorionated. They were desiccated for 7 min and pressure-injected with either the AS-2 solution in injection buffer [5 mM KCl and 100 mM sodium phosphate (pH 7.5)] or with buffer alone as a control. Batches of 20 embryos were injected, and at least three batches were injected for each concentration of AS-2 and the control. After injection, the embryos were developed for 20 to 30 min at room temperature inside a moist chamber and were fixed, devitelinized, immunostained for tubulin (Fig. 3, C, D, and F), and counterstained with 4',6'-diamidino-2-phenylindole (DAPI) (0.01 mg/ml) (Fig. 3, A through D and F). Images were recorded on a Bio-Rad MRC-1024 confocal laser scanning microscope using LaserSharp software.
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The Involvement of Cell-to-Cell Signals in the Development of a Bacterial Biofilm

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Bacteria in nature often exist as sessile communities called biofilms. These communities develop structures that are morphologically and physiologically differentiated from free-living bacteria. A cell-to-cell signal is involved in the development of *Pseudomonas aeruginosa* biofilms. A specific signaling mutant, a *lasI* mutant, forms flat, undifferentiated biofilms that unlike wild-type biofilms are sensitive to the biocide sodium dodecyl sulfate. Mutant biofilms appeared normal when grown in the presence of a synthetic signal molecule. The involvement of an intercellular signal molecule in the development of *P. aeruginosa* biofilms suggests possible targets to control biofilm growth on catheters, in cystic fibrosis, and in other environments where *P. aeruginosa* biofilms are a persistent problem.

Certain bacteria, such as the fruiting bacteria, communicate with each other to form structured macroscopic groups (1, 2). Recently, it has become apparent that in appropriate environments, common bacteria exhibit similar social behavior. Microscope observations of living bacterial biofilms attached to a glass surface have revealed that

these sessile microbial biofilm populations have a complicated structural architecture (3, 4). Biofilms of mixed bacterial communities and of individual species such as *Pseudomonas aeruginosa* that develop on solid surfaces exposed to a continuous flow of nutrients form thick layers consisting of differentiated mushroom- and pillar-like structures separated by water-filled spaces. The structures consist primarily of an extracellular polysaccharide (EPS) matrix or glycocalyx in which the bacterial cells are embedded (5). The finding that *P. aeruginosa* produces at least two extracellular signals involved in cell-to-cell communication and cell density-dependent expression of many secreted virulence factors suggests

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cell-to-cell signaling could be involved in the differentiation of *P. aeruginosa* biofilms, such as cell-to-cell signaling is involved in the development of specialized structures of fruiting bacteria like *Myxococcus* (1, 2). Thus, we initiated this study of the role of intercellular signals in *P. aeruginosa* biofilm development.

The two cell-to-cell signaling systems identified in *P. aeruginosa* are the *lasR-lasI* and *rhlR-rhlI* (also called *vsrR-vsrI*) systems (6–10). The *lasI* gene product directs the synthesis of a diffusible extracellular signal, *N*-(3-oxododecanoyl)-L-homoserine lactone (3OC₁₂-HSL). The *lasR* product is a transcriptional regulator that requires sufficient levels of 3OC₁₂-HSL to activate a number of virulence genes, including *lasI*, and the *rhlR-rhlI* system (11–14). The *rhlI* product directs the synthesis of the extracellular signal, *N*-butyryl-L-homoserine lactone, which is required for activation of virulence genes and expression of the stationary-phase σ factor, RpoS, by the *rhlR* gene product (13–16). At sufficient population densities these self-produced signals reach the concentrations required for gene activation. Thus, this type of gene regulation has been termed quorum sensing and response (17). Recently, acylhomoserine lactones have been detected in naturally occurring biofilms (18).

Because quorum sensing requires a sufficient density of bacteria, neither of the *P. aeruginosa* signals would be expected to participate in the initial stages of biofilm formation, attachment, and proliferation. However, these signals may be involved in biofilm differentiation. To test this hypothesis, we monitored biofilm formation of

wild-type (WT) *P. aeruginosa* PAO1 and a *lasI-rhlI* double mutant that makes neither of the quorum-sensing signals (19). Both strains adhered to and proliferated on the glass surface of the reaction chamber and reached a steady state within 2 weeks. However, the mutant biofilm was thin, about 20% of the WT thickness, and the cells were more densely packed (Fig. 1A). Furthermore, the WT formed characteristic microcolonies composed of groups of cells separated by water channels, whereas the mutant appeared to grow rather as continuous sheets on the glass surface. These results are consistent with the hypothesis that although not involved in the initial attachment and growth stages of biofilm formation, one or both of the *P. aeruginosa* quorum-sensing systems participates in the subsequent biofilm differentiation process.

To determine whether *lasI*, *rhlI*, or both are required for the normal development of the *P. aeruginosa* biofilm, we tested mutants defective in one or the other of these genes (19, 20). As indicated by measuring average thickness of biofilms and cell packing (Fig. 1A), the *rhlI* mutant formed biofilms similar to that of the WT, and the *lasI* mutant formed biofilms similar to that of the double mutant.

To further compare the WT and *lasI* mutant biofilms, we constructed a plasmid containing a gene encoding an enhanced green fluorescent protein (GFP) and introduced it into the two strains (21). This enabled us to image *P. aeruginosa* cells in the biofilms by epifluorescence and scanning confocal microscopy (Fig. 1B). Scanning confocal microscopy was used to produce a side view of the WT and mutant

biofilms. Only a few cells of an adherent cluster of the WT were apparent at the interface with the solid surface, and the cells appeared to be in a loose confederation with considerable intervening space between bacteria. Staining with alcian blue, which binds polysaccharides (22), showed that at least some of the intervening space consisted of an EPS matrix. The mutant biofilm was thin and much more uniform. A top view generated by epifluorescence microscopy showed the clusters of WT cells compared with the more uniform distribution of the *lasI* mutant (Fig. 1B).

To confirm that abnormal biofilm formation in the *lasI* mutant was due to the absence of 3OC₁₂-HSL, we added this compound to the medium flowing through a reaction chamber with a mutant biofilm (23). In the presence of 3OC₁₂-HSL, the *lasI* mutant formed biofilms of an average

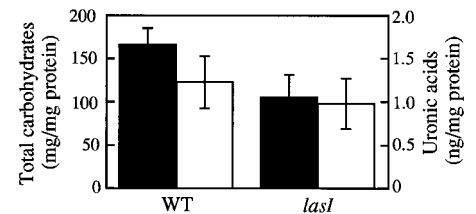


Fig. 2. Analysis of total carbohydrates (milligrams per milligram of total biofilm protein) and total uronic acids (nanograms per nanograms of total biofilm protein) in biofilm samples of the WT *P. aeruginosa*, PAO1, and the *lasI* mutant, PAO-JP1 (34). The filled bars show the average value for total carbohydrates, and the open bars show the average values for total uronic acids. The averages of two separate experiments are shown; bars correspond to the range.

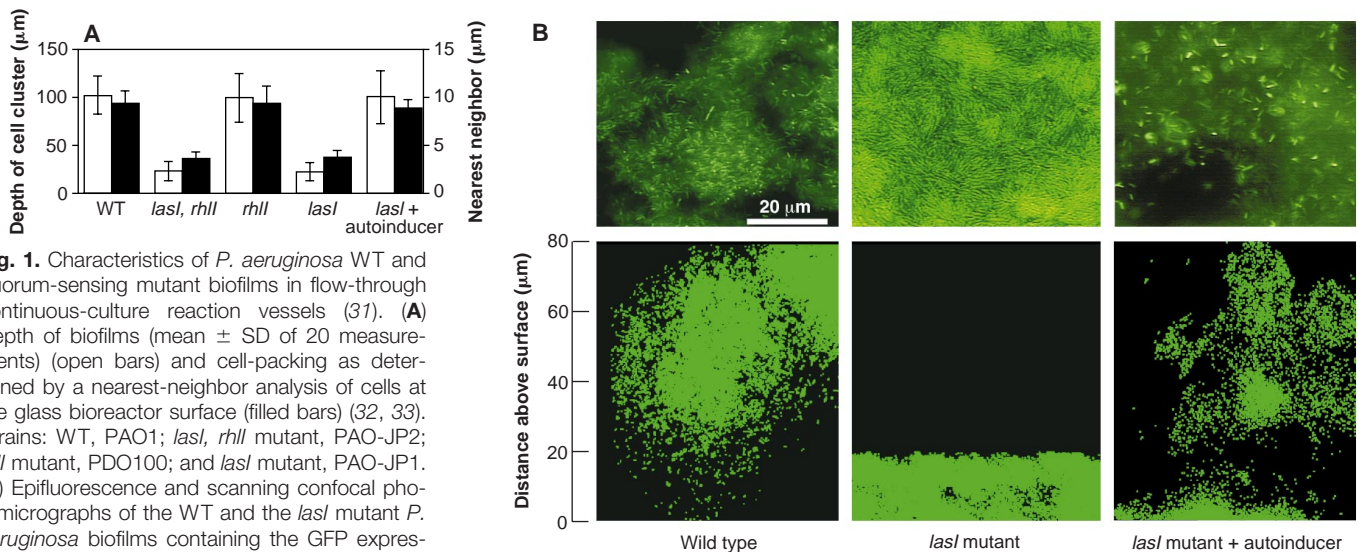


Fig. 1. Characteristics of *P. aeruginosa* WT and quorum-sensing mutant biofilms in flow-through continuous-culture reaction vessels (31). **(A)** Depth of biofilms (mean \pm SD of 20 measurements) (open bars) and cell-packing as determined by a nearest-neighbor analysis of cells at the glass bioreactor surface (filled bars) (32, 33). Strains: WT, PAO1; *lasI*, *rhlI* mutant, PAO-JP2; *rhlI* mutant, PDO100; and *lasI* mutant, PAO-JP1. **(B)** Epifluorescence and scanning confocal photomicrographs of the WT and the *lasI* mutant *P. aeruginosa* biofilms containing the GFP expression vector pMRP9-1. (Top) Epifluorescence photomicrographs of the WT (PAO1) and the *lasI* mutant (PAO-JP1) grown with or without the autoinducer, 3OC₁₂-HSL added to the medium. (Bottom) Sagittal views of Z series of wild-type and *lasI* mutant biofilms (with

or without 3OC₁₂-HSL) acquired by scanning confocal laser microscopy. Because the bacterial cells contain GFP, the color correlates with cell density.

thickness and cell density similar to that of the WT biofilms (Fig. 1A), and as shown by epifluorescence and confocal microscopy, the addition of the quorum-sensing signal allowed the development of clusters of relatively loosely packed cells (Fig. 1B). From this experiment we conclude that the quorum-sensing signal 3OC₁₂-HSL is required for normal biofilm differentiation, and that gradients of the signal do not appear to be necessary for this differentiation.

The EPS matrix is generally considered to be important in cementing bacterial cells together in the biofilm structure (24). The WT *P. aeruginosa* cells appeared to be embedded in an EPS matrix. Thus, we examined EPS levels in biofilms by measuring uronic acids (25), a constituent of the alginate EPS of *P. aeruginosa* (26), and by measuring total carbohydrates in biofilm samples (27). We detected no significant differences between the WT and the *lasI* mutant (Fig. 2) despite their markedly different appearance (Fig. 1). As shown previously, *P. aeruginosa* biofilm and free-floating (planktonic) cells also produce similar amounts of EPS (28). However, the distribution of the glycocalyx is different, with biofilm cells cemented to one another by the EPS matrix and planktonic cells having a compressed, incomplete glycocalyx (28). The mutant biofilms in our

study may have a glycocalyx matrix similar to that of planktonic cells. This could result in the tight packing of mutant biofilm bacteria. The results suggest that in the *lasI* mutant, the initial stages of biofilm formation proceed as normal, but differentiation from attached planktonic bacteria into biofilm bacteria does not proceed. Our hypothesis is that in the WT, this differentiation is triggered when the cell mass produces a sufficient amount of the quorum-sensing signal, 3OC₁₂-HSL. Although the signal generated by RhlI does not appear to participate in biofilm differentiation, there may well be other as yet unidentified signals implicated in this process.

Because we have hypothesized that the abnormal, undifferentiated biofilm formed by the *lasI* mutant contains cells similar in physiology to planktonic cells, we examined whether the abnormal mutant biofilm might be sensitive to biocides that do not disrupt WT biofilms. Thus, we exposed biofilms of the WT and the *lasI* mutant to the detergent sodium dodecyl sulfate (SDS, 0.2%). This treatment had no detectable effect on the WT, but within 5 min of SDS addition, most or all of the bacteria in the *lasI* mutant biofilm detached from the surface and dispersed (Fig. 3). Exposure of a *lasI* mutant biofilm grown in the presence of synthetic

3OC₁₂-HSL to 0.2% SDS for up to 24 hours had no detectable effect. As with WT biofilms, detachment and dispersal of the 3OC₁₂-HSL-rescued *lasI* mutant biofilm were not evident (Fig. 3), and indeed the average thickness of this biofilm was not changed by SDS treatment (93 ± 21 μm before and 24 hours after SDS treatment versus 102 ± 21 μm before and 24 hours after SDS treatment of the WT biofilm).

Our studies demonstrate that a cell-to-cell signal is required for the differentiation of individual cells of the common bacterium *P. aeruginosa* into complex multicellular structures. A mutation that blocks generation of the signal molecule hinders differentiation, and the resulting abnormal biofilm appears to be sensitive to the detergent biocide SDS. The control of biofilm differentiation and integrity by quorum sensing has important implications in medicine. *Pseudomonas aeruginosa* can colonize devices such as catheters (29), and it colonizes the lungs of most cystic fibrosis patients (30). Because of their innate resistance to antibiotics and other biocides, biofilms in these environments are difficult, if not impossible, to eradicate. Bacterial biofilms also present other problems of significant economic importance in both industry and medicine. Our finding of a connection between biofilm differentiation into clusters of bacteria resistant to the detergent biocide SDS and a quorum-sensing signal suggests that inhibition of these cell-to-cell signals could aid in the treatment of biofilms.

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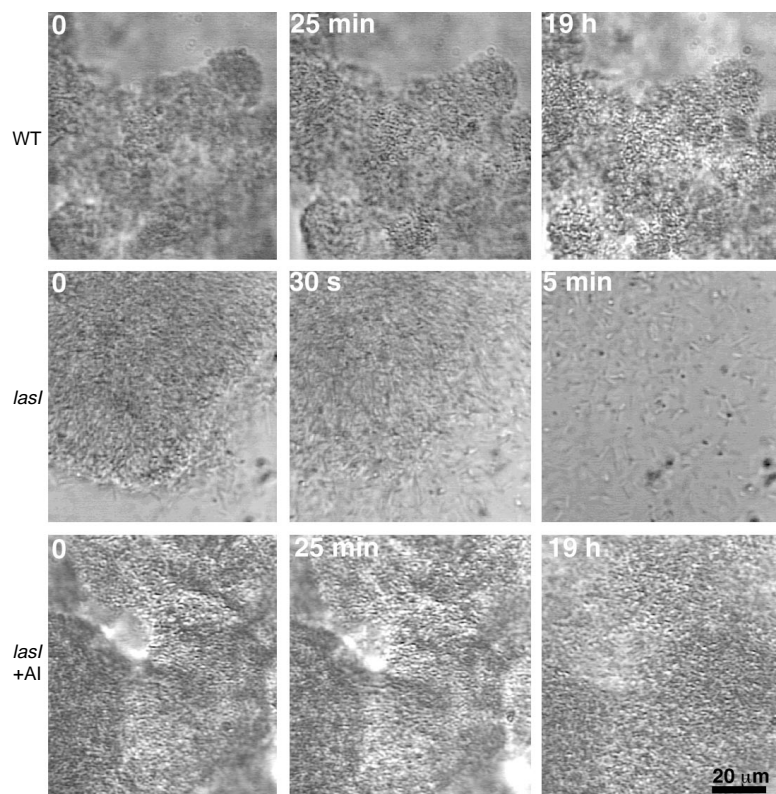


Fig. 3. SDS-induced detachment of mutant biofilm cells from a glass surface. Phase contrast photomicrographs of the *P. aeruginosa* WT strain, PAO1; the *lasI* mutant, PAO-JP1; and the *lasI* mutant grown in the presence of the autoinducer, 3OC₁₂-HSL (AI), immediately before addition of SDS, and at the times indicated after SDS addition (35).

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 31. All experiments were performed in EPRI medium, which contained 0.005% sodium lactate, 0.005% sodium succinate, 0.005% ammonium nitrate, 0.00019% KH₂PO₄, 0.00063% K₂HPO₄ (pH 7.0), 0.001% Hutner salts [G. Cohen-Bazire, W. R. Siström, R. Y. Stanier, *J. Cell. Comp. Physiol.* **49**, 35 (1957)], 0.1% glucose, and 0.001% L-histidine. Carbenicillin (150 μg/ml) was used to maintain pMRP9-1. A continuous-culture bioreactor was configured as a once-flow-through system [D. G. Davies, A. M. Chakrabarty, G. G. Geesey, *Appl. Environ. Microbiol.* **59**, 1181 (1993)]. Medium was pumped from the reservoir through the bioreactor at a flow rate of 0.13 ml/min. The flow was laminar with a Reynolds number of 0.17 and a fluid resistance time of 0.43 min. The bioreactor was composed of polycarbonate with a glass coverslip affixed to its top. This glass coverslip served as a substratum. *Pseudomonas aeruginosa* was introduced into the stream through a septum located 1 cm upstream of the bioreactor.
 32. An Olympus BH2 microscope with a 60× S PlanApo objective lens was used for epifluorescence and phase-contrast microscopy. Transmitted light images were collected with an Optronics charge-coupled device (Optronics Engineering, Goleta, CA) and the imaging program Image-Pro Plus 3.0 for Windows 95 (Media Cybernetics, Silver Spring, MD). Scanning confocal microscopy was performed with a Bio-Rad MRC600 confocal microscope (Hercules, CA). The excitation band was 488 nm with a 514-nm cutoff. The 50× ULWD Olympus objective lens was used for scanning confocal microscopy. The imaging software was Comos 7.0 (Bio-Rad). The images were constructed with the SLICER imaging program (Fortner Research, Sterling, VA). The Kalman for each cross section was 5.
 33. The nearest-neighbor analyses were on transmitted-light images of bacteria attached to the substratum after 10 days of continuous culture. A minimum of 3000 cells in 10 fields were analyzed for each population. The distance of the nearest cell centroid to each study cell centroid was measured, and the average nearest-neighbor distance was calculated. This analysis was developed by G. Harkin (Montana State University, Bozeman, MT).
 34. Biofilms for chemical analysis were grown in silicone

tubing in a once-through continuous-flow system. Size 15 silicone tubing with a flow rate of 0.13 ml/min or size 15 tubing with a flow rate of 0.8 ml/min was used. After biofilms matured, the tubing was sliced lengthwise and biofilm cells were collected. The collected material was centrifuged at 13,000 rpm (Eppendorf Microfuge) for 10 min and the sedimented material was analyzed for total carbohydrates, uronic acids, and protein as described (25, 27).

35. SDS (0.2% w/v) in 10 ml of EPRI medium was filtered through a 0.2-μm polycarbonate filter. After the flow of medium into the bioreactor was stopped, the SDS was injected through the septum into the flow chamber. The SDS remained in the bioreactor for 30 min, and then the flow of fresh medium was reinitiated.

36. We thank D. Ohman for providing the *rhlI* mutant PDO100, B. Cormack and S. Falkow for providing pGFPmut2, T. Moninger for assistance in preparation of the micrographs, A. Kende for synthetic 3OC₁₂-HSL, L. Loetterle for technical assistance, and T. de Kievit for helpful discussions. Supported by Office of Naval Research grant N00014-5-0190 and a grant from the Cystic Fibrosis Foundation (E.P.G.), NIH grant AI33713 (B.H.I.), and cooperative agreement ECD-8907039 with the Engineering Research Centers and Education Division of NSF (J.W.C.). M.R.P. is an NIH Postdoctoral Fellow (GM 18740), and J.P.P. is an NIH Predoctoral Trainee (5T32AI07362).

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Coupling Termination of Transcription to Messenger RNA Maturation in Yeast

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The direct association between messenger RNA (mRNA) 3'-end processing and the termination of transcription was established for the *CYC1* gene of *Saccharomyces cerevisiae*. The mutation of factors involved in the initial cleavage of the primary transcript at the poly(A) site (*RNA14*, *RNA15*, and *PCF11*) disrupted transcription termination at the 3' end of the *CYC1* gene. In contrast, the mutation of factors involved in the subsequent polyadenylation step (*PAP1*, *FIP1*, and *YTH1*) had little effect. Thus, cleavage factors link transcription termination of RNA polymerase II with pre-mRNA 3'-end processing.

Polyadenylation signals at the 3' end of pre-mRNA are required for the termination of transcription in higher eukaryotes (1), budding yeast (2), and fission yeast (3). This ensures that transcription will only terminate after RNA polymerase II (pol II) has read beyond the end of the mRNA sequence. The mechanism of pre-mRNA 3'-end formation (sequential endonucleolytic cleavage and polyadenylation) and many of the factors involved in catalyzing these reactions are very similar between higher eukaryotes and *S. cerevisiae* (4). Reconstitution of this reaction in vitro in *S. cerevisiae* has allowed the fractionation of factors [cleavage factor IA (CF IA), CF IB, CF II, polyadenylation factor I (PF I), and poly(A) polymerase (PAP)] required for each step in the 3'-end formation reaction (5). The use of temperature-sensitive (ts) mutants has facilitated the determination of the molecular composition of these factors and revealed interactions between them (6). Here, we used yeast strains carrying

these ts mutant alleles to demonstrate that some of these factors are also involved in pol II transcription termination.

We used transcription run-on analysis to measure transcription termination at the 3' end of the *S. cerevisiae* *CYC1* gene (Fig. 1), for which signals that direct 3'-end formation have been well characterized (7). To achieve high transcription, we transformed yeast cells (8) with the multicopy plasmid pGCYC, in which the *CYC1* promoter has been replaced by the *GAL1/10* promoter region (9). Reverse transcription polymerase chain reaction (RT-PCR) analysis confirmed that transcripts initiating at the *GAL* promoter are polyadenylated at the same sites as found for the intact *CYC1* gene (10, 11). The distribution of run-on transcript over the contiguous single-stranded probes 1 to 6 showed that transcription stops efficiently, soon after the *CYC1* poly(A) site (located at the 3' end of probe 2), with only small amounts of run-on transcript detected beyond probe 3 (Fig. 1, B and D). This finding is in agreement with previous in vivo data showing that signals 100 base pairs (bp) beyond the *CYC1* poly(A) site are required to direct the termination of transcription (2). The background signal detected in the upstream *GAL* probe indicates that transcription begins at the *GAL* promoter. A similar distribution of polymerases was observed with the genomic copy of the *CYC1* gene, al-

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