EUKARYOTIC CELL, Oct. 2006, p. 1770–1779 Vol. 5, No. 10
1535-9778/06/$08.00+0 doi:10.1128/EC.00219-06

Production of Tyrosol by *Candida albicans* Biofilms and Its Role in Quorum Sensing and Biofilm Development[▽]

Mohammed A. S. Alem,[1] Mohammed D. Y. Oteef,[2] T. Hugh Flowers,[2] and L. Julia Douglas[1]*

Division of Infection and Immunity, Institute of Biomedical and Life Sciences,[1] and Department of Chemistry,[2] University of Glasgow, Glasgow G12 8QQ, United Kingdom

Received 12 July 2006/Accepted 8 August 2006

Tyrosol and farnesol are quorum-sensing molecules produced by *Candida albicans* which accelerate and block, respectively, the morphological transition from yeasts to hyphae. In this study, we have investigated the secretion of tyrosol by *C. albicans* and explored its likely role in biofilm development. Both planktonic (suspended) cells and biofilms of four *C. albicans* strains, including three mutants with defined defects in the Efg 1 and Cph 1 morphogenetic signaling pathways, synthesized extracellular tyrosol during growth at 37°C. There was a correlation between tyrosol production and biomass for both cell types. However, biofilm cells secreted at least 50% more tyrosol than did planktonic cells when tyrosol production was related to cell dry weight. The addition of exogenous farnesol to a wild-type strain inhibited biofilm formation by up to 33% after 48 h. Exogenous tyrosol appeared to have no effect, but scanning electron microscopy revealed that tyrosol stimulated hypha production during the early stages (1 to 6 h) of biofilm development. Experiments involving the simultaneous addition of tyrosol and farnesol at different concentrations suggested that the action of farnesol was dominant, and 48-h biofilms formed in the presence of both compounds consisted almost entirely of yeast cells. When biofilm supernatants were tested for their abilities to inhibit or enhance germ tube formation by planktonic cells, the results indicated that tyrosol activity exceeds that of farnesol after 14 h, but not after 24 h, and that farnesol activity increases significantly during the later stages (48 to 72 h) of biofilm development. Overall, our results support the conclusion that tyrosol acts as a quorum-sensing molecule for biofilms as well as for planktonic cells and that its action is most significant during the early and intermediate stages of biofilm formation.

Quorum-sensing or population-dependent gene expression is a well-known cell signaling mechanism in bacteria and is also thought to be significant in biofilm formation (8, 23, 27). Related quorum-sensing systems have now been described for fungal species (5, 11, 20). In gram-negative bacteria, acylated homoserine lactones are signal molecules that accumulate in bacterial cultures as a function of cell density. At a threshold population density, described as a quorum, the accumulated signal molecules interact with cellular receptors that control the expression of a set of specific target genes. The expression of these genes is therefore regulated in response to local cell density. Gram-positive bacteria do not synthesize acylated homoserine lactones but instead produce peptide signal molecules.

The fungal pathogen *Candida albicans* was the first eukaryotic microorganism shown to exhibit quorum sensing. This organism is an important agent of hospital-acquired infections, many of which involve the formation of an adherent population or biofilm on implanted devices, such as catheters and prosthetic heart valves (9). *C. albicans* has the ability to switch from yeast morphology to hyphal morphology, and this characteristic is thought to be a major determinant of virulence. The quorum-sensing signal molecule identified in *C. albicans* was not an acyl homoserine lactone or a peptide but the sesquiterpene farnesol (3,7,11-trimethyl-2,6,10-dodecatriene-1-ol). The accumulation of farnesol blocks the morphological shift from yeasts to hyphae at high cell densities, and exogenously added farnesol inhibits germ tube formation as normally triggered by serum, proline, or *N*-acetylglucosamine (11). Farnesol blocks germ tube formation but does not prevent the elongation of existing germ tubes (18). In one strain of *C. albicans*, farnesoic acid, rather than farnesol, appears to fulfill a similar quorum-sensing role (22).

Tyrosol (2-[4-hydroxyphenyl] ethanol), a derivative of tyrosine, was subsequently identified as a second quorum-sensing molecule in *C. albicans* (5). Like farnesol, this compound is released into the growth medium continuously during growth and is capable of abolishing the lag phase normally seen when overnight cultures are diluted into fresh medium. Tyrosol accelerates the formation of germ tubes. It appears, therefore, that morphogenesis in *C. albicans* is under complex positive and negative control by the actions of tyrosol and farnesol, respectively (5). Recently, the aromatic alcohols phenylethanol and tryptophol were identified as quorum-sensing molecules in *Saccharomyces cerevisiae* (6). These compounds, which are also produced by *C. albicans* (6, 15), stimulated pseudohyphal growth in *S. cerevisiae* at relatively low concentrations, whereas tyrosol had no effect.

The high density of microorganisms within biofilms led to speculation that quorum sensing may play an important role in biofilm-specific physiology. A connection between quorum sensing and biofilm development has now been demonstrated in several bacterial species. In some bacteria, including *Pseudo-*

* Corresponding author. Mailing address: Division of Infection and Immunity, Institute of Biomedical and Life Sciences, Joseph Black Building, University of Glasgow, Glasgow G12 8QQ, United Kingdom. Phone: 44 141 330 5842. Fax: 44 141 330 4600. E-mail: J.Douglas@bio.gla.ac.uk.

▽ Published ahead of print on 15 September 2006.

monas aeruginosa and the emerging pathogen *Burkholderia cepacia*, quorum sensing appears to be involved in the formation of normal biofilm structures (8, 12). For example, mutants of *P. aeruginosa* that are deficient in the synthesis of the acyl-homoserine lactone signal molecule form thin, undifferentiated biofilms in contrast to the highly structured biofilms of wild-type strains (8). In other bacteria, quorum sensing may function in the dispersal of individual organisms from the biofilm (23, 24). Biofilms of *C. albicans* formed on disks cut from polyvinyl chloride catheters consist of a dense network of yeasts, hyphae, and pseudohyphae (9). There are two distinct layers: a thin, basal region of densely packed yeast cells that appears to anchor the biofilm to the surface and an overlying thicker, mainly hyphal layer. Morphogenesis is not an absolute prerequisite for biofilm formation but seems to be necessary for the development of a spatially organized structure (9). Exogenous farnesol inhibits biofilm formation in *C. albicans* when added at an early stage in the process. Cells treated with farnesol at a concentration of 300 μM produce sparse biofilms composed predominantly of yeasts and pseudohyphae (25).

In this study, we have investigated the production of tyrosol by biofilms of *C. albicans*, including strains defective in yeast-hypha morphogenesis. We show that tyrosol production correlates with biofilm cell dry weight, supporting the conclusion that tyrosol acts as a quorum-sensing molecule for biofilms as well as for planktonic cells. In addition, we have monitored the effect of tyrosol on the formation of hyphae during the early stages of biofilm development and we have explored possible interactions between farnesol and tyrosol in regulating the growth of adherent cell populations.

MATERIALS AND METHODS

Organisms. Five strains of *C. albicans* were used in this study. *C. albicans* GDH 2346 was originally isolated at Glasgow Dental Hospital from a patient with denture stomatitis. *C. albicans* SC5314 (wild type) and its isogenic mutants JKC19 (*cph1*/*cph1*), HLC52 (*efg1*/*efg1*), and HLC54 (*cph1*/*cph1 efg1*/*efg1*) (16) were kindly provided by N. A. R. Gow, University of Aberdeen, Aberdeen, Scotland, with permission from G. R. Fink. All strains were maintained on slopes of Sabouraud dextrose agar (Difco).

Medium and culture conditions. Organisms were grown in yeast nitrogen base (YNB) medium (Difco) with 50 mM glucose. Batches of medium (25 ml, in 100-ml Erlenmeyer flasks) were inoculated from fresh culture slopes and incubated at 37°C in an orbital shaker at 60 rpm. All strains grew exclusively in the budding yeast phase under these conditions. Cells were harvested after 24 h and washed twice in 0.15 M phosphate-buffered saline (PBS), pH 7.2. Before use in biofilm experiments, washed cell suspensions were standardized to an optical density of 0.8 at 520 nm.

Tyrosol and farnesol. Stock solutions (100 mM) of tyrosol (2-[4-hydroxyphenyl] ethanol; Sigma-Aldrich) were prepared in distilled water; tyrosol was used at final concentrations ranging from 20 μM to 1 mM. Stock solutions (100 mM) of farnesol (*E*,*E* farnesol; Sigma) were prepared in methanol. In biofilm experiments, farnesol was used at final concentrations of 50 μM, 100 μM, and 1 mM.

Biofilm formation on catheter disks. Biofilms were grown on small disks (surface area, 0.5 cm^2) cut from polyvinyl chloride Faucher tubes (French gauge 36; Vygon, Cirencester, United Kingdom) as described previously (2, 10). Briefly, the disks were placed in the wells of 24-well Nunclon tissue culture plates and a standardized cell suspension (80 μl) was applied to the surface of each one. Initially, the incubation lasted for 1 h at 37°C (adhesion period). Nonadherent organisms were removed by washing, and the disks were then incubated for a further 48 h at 37°C while they were submerged in 1 ml of growth medium (biofilm formation). Unless stated otherwise, tyrosol or farnesol was added at the beginning of the 1-h adhesion period and again at time zero of the subsequent 48-h incubation. Controls included disks with no cells, disks with cells and solvent but no tyrosol or farnesol, and disks with tyrosol or farnesol but no cells. At the end of the incubation period, disks were gently washed twice with PBS and transferred to fresh 24-well plates for quantitative measurement of biofilm formation.

Quantitative measurement of biofilm formation. Biofilm growth was quantified colorimetrically by a 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay. XTT solution (250 μg ml^{-1}; Sigma) was prepared in PBS containing 1% (wt/vol) glucose, and 1 ml was added to each well of the fresh plates containing the biofilm disks. Menadione solution (4 μl; 1 mM in acetone; Sigma) was also added to the wells, and the plates were incubated for 5 h at 37°C in the dark. After incubation, the liquid was removed from each well, transferred to a microfuge tube, and clarified by centrifugation at 13,000 rpm and XTT formazan production was measured by determining the absorbance at 492 nm. Control disks were used to check for interference with XTT reduction by tyrosol or farnesol.

Preparation of culture supernatants for tyrosol determinations. For planktonic cultures, standardized cell suspension (100 μl) was used to inoculate YNB medium (50 ml in 250-ml Erlenmeyer flasks), giving a cell density of 4×10^4 cells ml^{-1} as measured in a hemocytometer. Cultures were incubated at 37°C in an orbital shaker at 60 rpm for time periods of up to 48 h and then centrifuged at 3,000 rpm. The supernatants were decanted and filter sterilized through a 0.2-μm filter. Biofilms were grown on the surfaces of 75-cm^2 flat-bottomed tissue culture flasks (Costar; Corning Incorporated, Corning, NY) with vented caps. Standardized cell suspensions of *C. albicans* strains were prepared as described above. Cell suspension (10 ml) was added to the surface of each tissue culture flask, and the cells were allowed to adhere for 1 h at 37°C. The remaining cell suspension was decanted, and nonadherent cells were removed by washing with 10 ml of PBS. Fresh YNB (110 ml) was added, and the biofilm was grown over 48 h at 37°C under static conditions. The biofilm supernatant was then decanted, centrifuged at 3,000 rpm, and filter sterilized using a 0.2-μm filter.

Dry weight measurements for tyrosol determinations. At the end of the incubation period, 3 ml of planktonic cells or resuspended biofilm cells was collected on preweighed cellulose nitrate filters (0.45-μm pore size; 25-mm diameter; Whatman) and given three washes with water (5 ml). The filters were dried to constant weight at 37°C, and the dry weight of cells on each filter was calculated. Dry weights were determined in triplicate.

Germ tube formation. To investigate the ability of culture supernatants to inhibit or enhance germ tube formation, cultures of *C. albicans* SC5314 grown overnight in YNB-glucose medium were harvested and the cells were washed twice in 50 mM potassium phosphate buffer, pH 6.5. The cells were then resuspended at 10^7 cells ml^{-1} in 2.5 ml of a twofold concentrate of the same buffer containing 10 mM proline and 2.5 mM *N*-acetylglucosamine. This suspension was added to 2.5 ml of filter-sterilized supernatant from biofilms or planktonic cultures. For a standard response curve, cells were resuspended at 10^7 cells ml^{-1} in 5 ml of same buffer containing 10 mM proline and 2.5 mM *N*-acetylglucosamine with farnesol at final concentrations ranging from 5 to 100 μM. Cell suspensions were incubated with gentle shaking at 37°C for 3 h. At 0 min and 3 h, the suspensions were examined for the percentage of germ tubes present using a light microscope; 200 cells were counted each time.

Scanning electron microscopy. Biofilms formed on catheter disks, or on polystyrene disks (surface area, 0.5 to 0.6 cm^2) cut from flat-bottomed 75-cm^2 tissue culture flasks, were treated with glutaraldehyde and osmium tetroxide as described previously (10). After dehydration in a series of ethanol solutions, samples were dried overnight in a desiccator, coated with gold using a Polaron coater, and viewed with a Joel 6400 scanning electron microscope.

HPLC. Tyrosol was isolated from culture supernatants using solid-phase extraction (SPE) and quantified by reverse-phase high-pressure liquid chromatography (HPLC). The method was a modification of that recently described by Chen et al. (5). A 1 mM stock solution of tyrosol in acetonitrile was prepared and diluted to give working standards. For SPE, culture supernatant (50 ml) was acidified by the addition of 0.2 ml of 0.1 M sulfuric acid. A C-18 Sep-Pak Plus cartridge (packed with 820 mg silica-based Sep-Pak; Waters) was conditioned with 10 ml of methanol (HPLC grade; Fisher Scientific, United Kingdom), followed by 10 ml of 1 mM sulfuric acid (AnalaR grade; BDH, United Kingdom). The acidified supernatant was then loaded onto the cartridge. Tyrosol was eluted with 10 ml of 7.5% acetonitrile in 1 mM sulfuric acid. The loading and eluting flow rates were controlled to be 2 to 4 ml min^{-1} in an SPE vacuum manifold. The HPLC system used consisted of a Merck-Hitachi L7100 pump, an L7200 autosampler, and an L4500 diode array detector. The signal and UV spectra were processed by Merck-Hitachi chromatography data station software. HPLC analyses were carried out using a C-18 Spherisorb ODS2 analytical column (4.6 by 250 mm; 5-μm particles; Waters). The elution profile consisted of three mobile-phase compositions over 30 min of run time. For the first 10 min, the mobile phase consisted of acetonitrile-1 mM H_2SO_4 (10:90, vol/vol) for eluting tyrosol. The mobile-phase composition for the second 10 min was acetonitrile-water

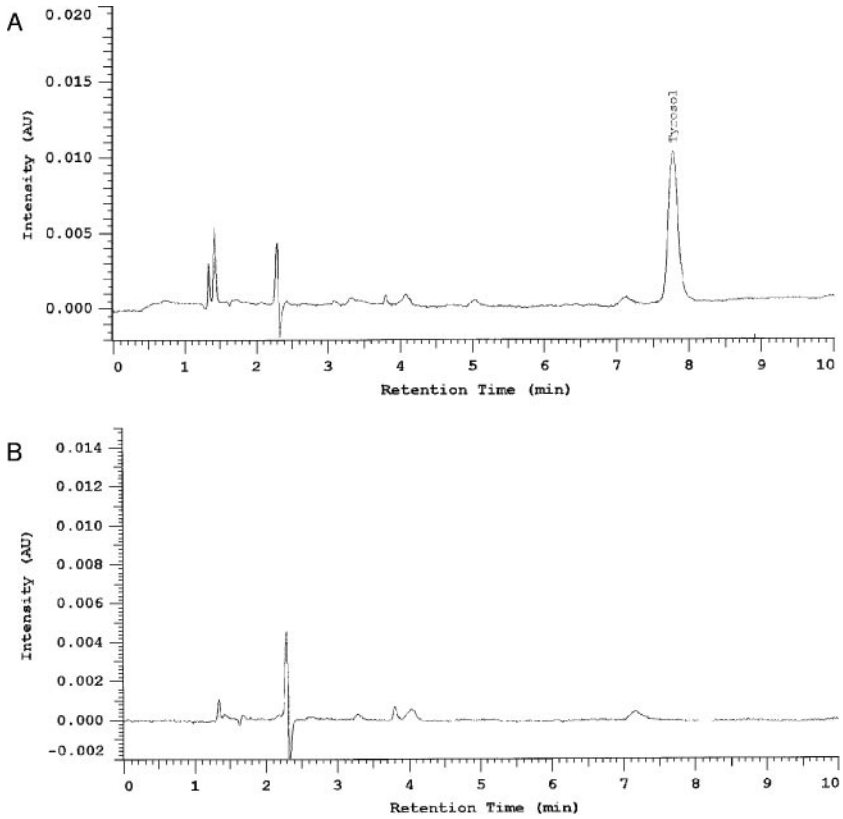

FIG. 1. Typical HPLC chromatograms for the determination of tyrosol. (A) A supernatant extract containing 7 μM tyrosol. (B) Uninoculated, extracted growth medium showing no interfering peaks at or around the tyrosol retention time (7.7 min).

(50:50, vol/vol) to clean up the column after some late-eluted compounds. The column was then reconditioned using acetonitrile-1 mM H_2SO_4 (10:90, vol/vol) for 10 min to be ready for the next injection. The flow rate was set at 1.5 ml min^{-1}, and the analysis was carried out at room temperature. An aliquot (10 μl) of the purified extract was injected into the HPLC system. The photodiode array was programmed to record data from 200 to 400 nm.

HPLC method validation. The tyrosol peaks were identified, and their purities were checked by comparing their spectra with those of a pure standard. The amount of tyrosol present was quantified by external calibration in the range of 0 to 100 μM. The system precision was examined by analyzing replicate injections ($n = 7$) at the targeted limit of quantification (1 μM) and at the usual working level (50 μM) and gave relative standard deviation values of 7.0 and 0.8%, respectively. The recovery of the SPE method was assessed by spiking replicate samples with 6 μM tyrosol. This evaluation confirmed that the method has high levels of accuracy and precision, with a 92.8% ± 0.8% recovery of tyrosol. The growth medium was analyzed for possible interference and was shown to have no interfering substances eluting at or near the retention time of tyrosol (Fig. 1).

RESULTS

Tyrosol production by biofilms and planktonic cells of *C. albicans*. Tyrosol production was determined during growth of both planktonic cells and biofilms of four strains of *C. albicans*. The strains used were *C. albicans* SC5314 (wild type) and its isogenic mutants JKC19 (*cph1*/*cph1*), HLC52 (*efg1*/*efg1*), and HLC54 (*cph1*/*cph1 efg1*/*efg1*), all of which have defined defects in filamentation pathways (16). Planktonic cells were grown in shake flasks and biofilms in flat-bottomed tissue culture flasks, and tyrosol concentrations in culture supernatants were measured at intervals over 48 h. For planktonic cells, tyrosol was undetectable at 5 h, but after 10 h, it had reached concentrations ranging from 0.09 to 1.28 μM (Fig. 2). Tyrosol production by all strains then increased rapidly to a concentration of at least 11.6 ± 1.3 μM (mean ± standard error of the mean [SEM]) after 48 h. Results obtained for biofilms were similar (Fig. 3). There was a sharp increase in tyrosol concentration between 10 h and 24 h, followed by a leveling off to 9.3 to 9.6 μM for most strains. However, tyrosol production by *C. albicans* HLC52 continued to increase to 13.6 μM after 48 h; the reasons for the different behavior of this strain are not known. For both planktonic cells and biofilms, there was a good correlation between tyrosol formation and biomass (Fig. 2 and 3).

When tyrosol production was calculated as a function of cell dry weight (Fig. 4), it became clear that biofilm cells produce significantly more tyrosol than do planktonic cells. For example, tyrosol production at 48 h by biofilms of *C. albicans* SC5314 and JKC19 was 9.6 ± 0.5 and 9.7 ± 0.3 nmol/mg (dry weight), respectively, whereas for planktonic cells, it was 5.7 ± 1.1 and 6.3 ± 1.5 nmol/mg (dry weight), respectively ($P < 0.05$) (Fig. 4A and B). With biofilms of *C. albicans* HLC52 and HLC54, tyrosol production was also significantly greater than that of planktonic cells (Fig. 4C and D). At 24 and 48 h, for example, tyrosol production by biofilms of *C. albicans* HLC52 was 10.1 ± 1.8 and 16.4 ± 3.2 nmol/mg (dry weight), respectively, which is substantially more than that for planktonic cells (3.9 ± 0.6 and 6.0 ± 0.3 nmol/mg (dry weight), respectively) at the same time points ($P < 0.05$) (Fig. 4C).

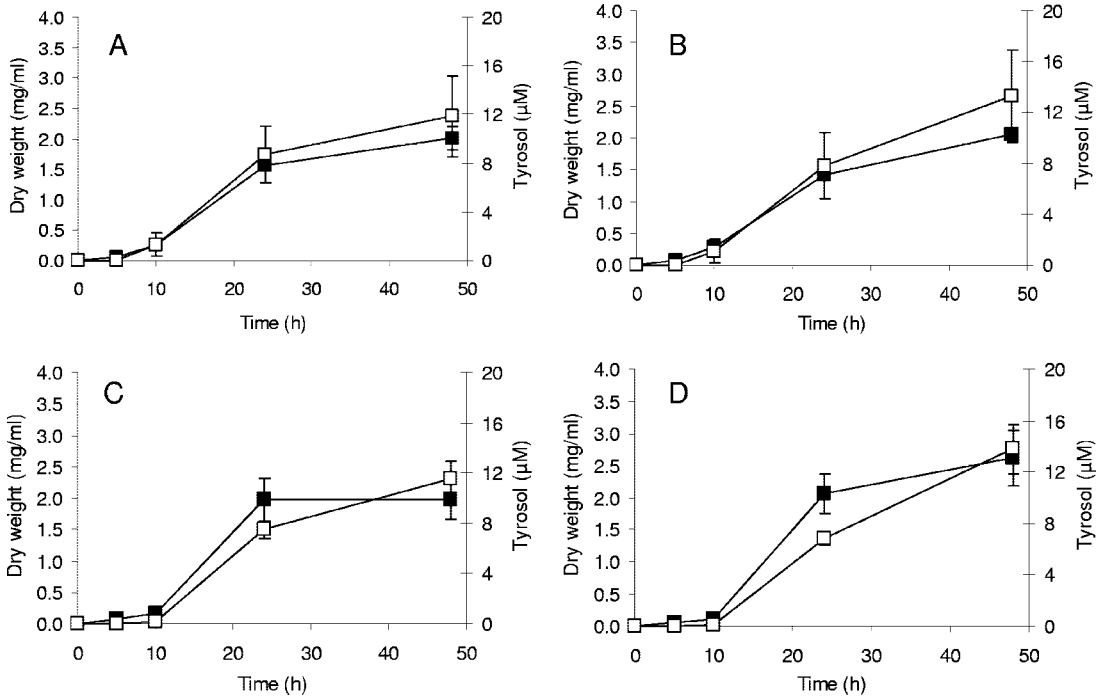

FIG. 2. Relationship between tyrosol production (□) and cell dry weight (■) for planktonic cells of *C. albicans* strains SC5314 (A), JKC19 (B), HLC52 (C), and HLC54 (D). Results are means ± SEMs of three independent experiments.

Biofilm formation in the presence of exogenous tyrosol or farnesol. Previous studies have shown that farnesol prevents hyphal formation and biofilm development by *C. albicans* (25), whereas, conversely, tyrosol promotes hyphal formation (5). Farnesol at three different concentrations (50 μM, 100 μM, and 1 mM) was added at different stages of biofilm production by *C. albicans* GDH 2346 in YNB medium. Only the early stages were sensitive to farnesol (Fig. 5), i.e., when farnesol

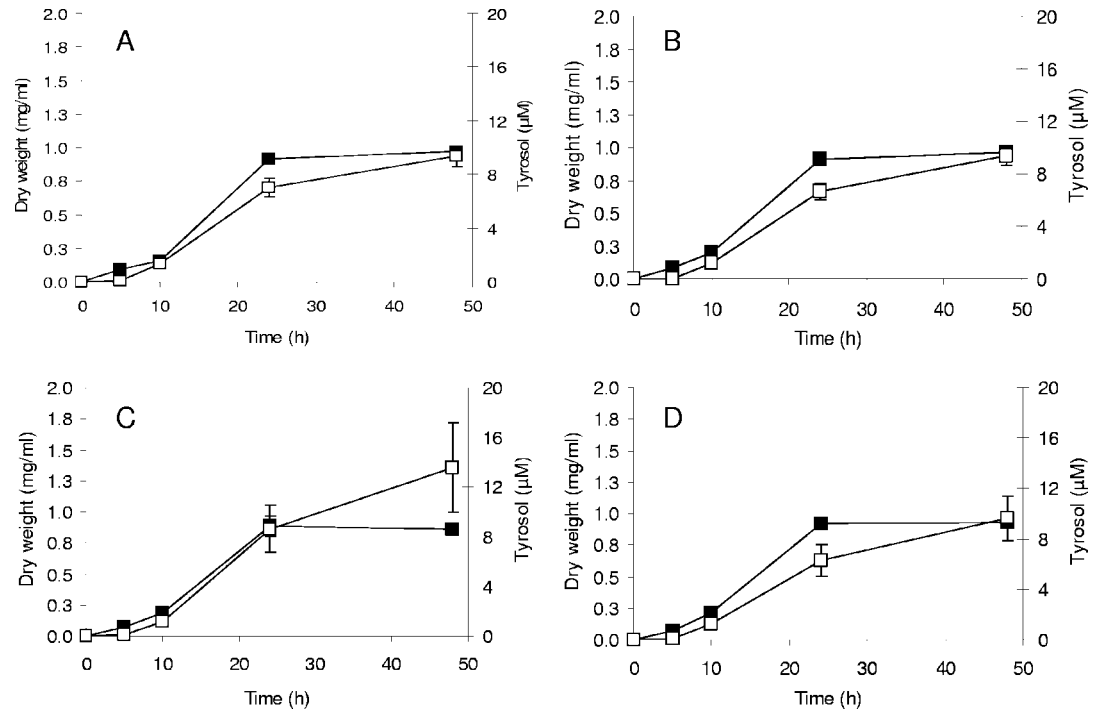

FIG. 3. Relationship between tyrosol production (□) and cell dry weight (■) for biofilms of *C. albicans* SC5314 (A), JKC19 (B), HLC52 (C), and HLC54 (D). Results are means ± SEMs of three independent experiments.

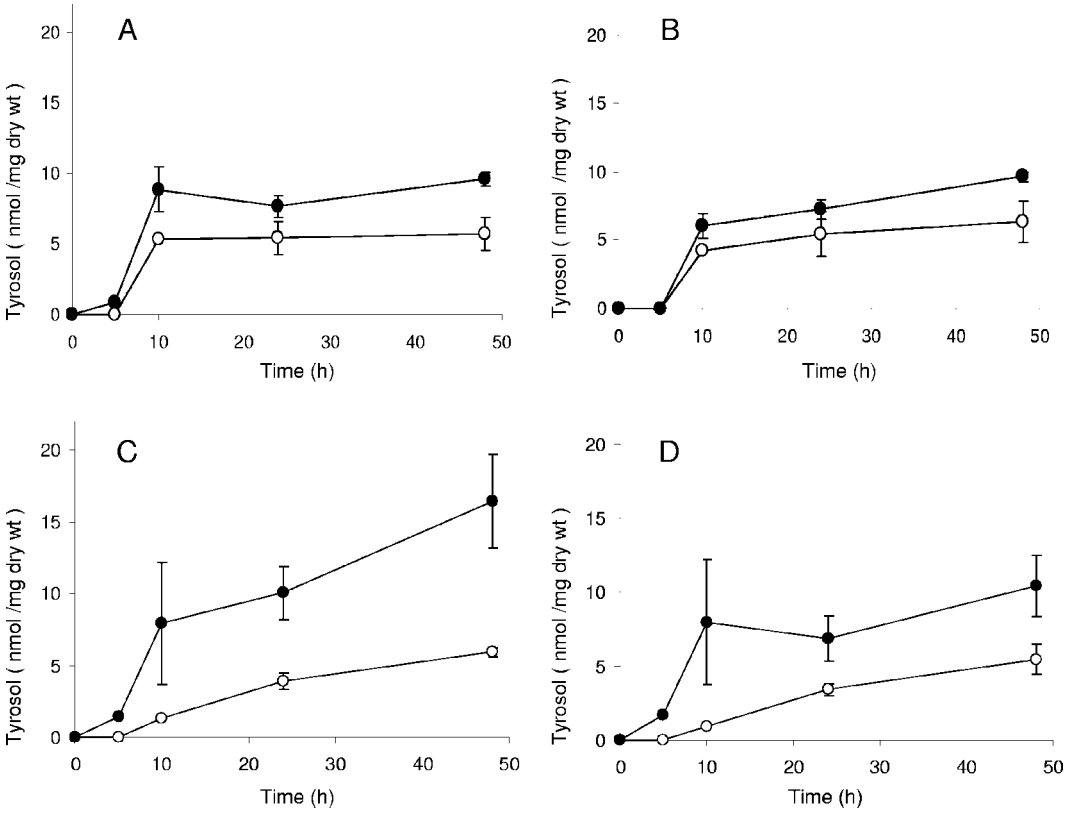

FIG. 4. Tyrosol production expressed as a function of cell dry weight for planktonic cells (○) and biofilms (●) of *C. albicans* SC5314 (A), JKC19 (B), HLC52 (C), and HLC54 (D). Results are means ± SEMs of three independent experiments carried out in triplicate.

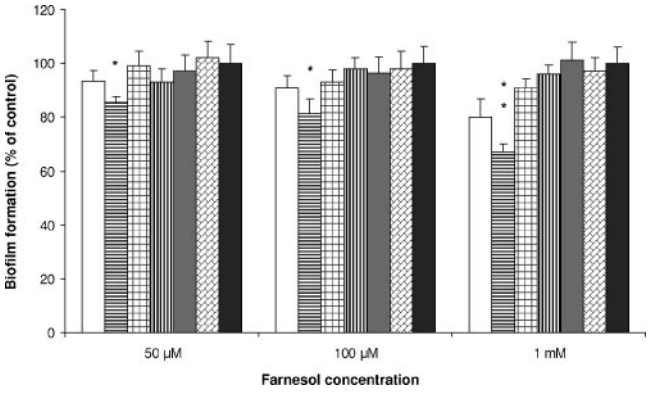

FIG. 5. Effect of farnesol concentration on biofilm formation by *C. albicans* GDH 2346. Farnesol, at concentrations of 50 μM, 100 μM, and 1 mM, was added at different stages of biofilm development. Biofilm formation (XTT reduction) is expressed as a percentage of that of control biofilms incubated in the absence of farnesol. Results are means ± SEMs from at least two independent experiments carried out in triplicate. Mean (±SEM) control values (A_{492}) ranged from 1.999 ± 0.399 to 2.311 ± 0.142. Farnesol was added at adhesion (□) adhesion and time zero (▤) time zero (▦), 2 h of biofilm formation (▥), 4 h of biofilm formation (▤), and 24 h of biofilm formation (▨). ■, control biofilm (no farnesol added). *P* was <0.05 (*) and <0.001 (**) for treated biofilms compared with untreated controls (Student's *t* test).

was added during the adhesion period and again at time zero, so that it was present throughout biofilm formation. Farnesol at 50 and 100 μM inhibited biofilm formation by 14 and 19% ($P < 0.05$), respectively, when added at this stage. Maximal inhibition of 33% ($P < 0.001$) was observed with 1 mM farnesol again when added during the adhesion period and maintained throughout biofilm formation (Fig. 5). In contrast, analogous experiments with tyrosol failed to show any effect. When 20 or 50 μM tyrosol was added at different stages (0, 3, 6, and 24 h), biofilm formation after 48 h was similar to that of the controls (results not shown). Moreover, higher tyrosol concentrations (100 μM, 500 μM, and 1 mM) added during the adhesion period and again at time zero also had no significant effect on biofilm formation (Fig. 6).

Effect of simultaneous addition of tyrosol and farnesol on biofilm formation. To determine whether the inhibitory effect of farnesol on biofilm formation could be abolished by tyrosol, different concentrations of tyrosol (100 μM, 500 μM, and 1 mM) were added together with different concentrations of farnesol (50 μM, 100 μM, and 1 mM) for the adhesion period and throughout the 48-h incubation. All of the tyrosol concentrations abolished the effect of 50 μM farnesol which, on its own, inhibited biofilm formation by 15% ($P < 0.05$) (Fig. 6). The effect of 100 μM farnesol was abrogated only by 1 mM tyrosol. Farnesol at 1 mM inhibited biofilm formation by 29%, and none of the three tyrosol concentrations tested was able to counteract this inhibitory effect (Fig. 6).

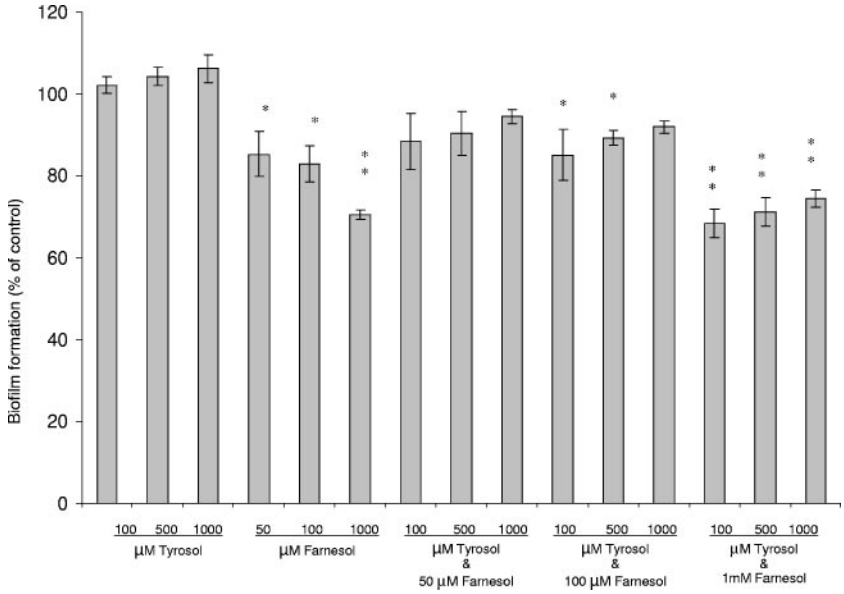

FIG. 6. Effect of simultaneous addition of farnesol and tyrosol on biofilm formation by *C. albicans* GDH 2346. Farnesol and tyrosol were present during the adhesion period and throughout the 48-h incubation. Biofilm formation (XTT reduction) is expressed as a percentage of that of control biofilms incubated in the absence of farnesol or tyrosol. Results are means ± SEMs from two independent experiments performed in duplicate. The mean (±SEM) control value for biofilm formation in the absence of farnesol (A_{492}) was 2.477 ± 0.099. *P* was <0.05 (*) and <0.001 (**) for treated biofilms compared with untreated controls (Student's *t* test).

Scanning electron microscopy of biofilm formation in the presence of tyrosol and/or farnesol. Biofilms of *C. albicans* SC5314 were formed on polystyrene disks cut from 75-cm^2 tissue culture flasks (to match the procedure used for tyrosol detection by HPLC) over 48 h in the presence of 500 μM tyrosol and/or 1 mM farnesol. Scanning electron microscopy showed that farnesol-treated biofilms consisted of yeast cells only (Fig. 7B), while control biofilms or tyrosol-treated biofilms contained a mixture of yeasts and hyphae (Fig. 7A and D). Biofilms formed in the presence of both farnesol and tyrosol, on the other hand, consisted almost exclusively of yeasts (Fig. 7C). This indicates that tyrosol did not counteract the effect of farnesol in preventing hyphal formation at these concentrations. Tyrosol alone also had no effect on the morphologies of biofilms produced by the mutant strains JKC19, HLC52, and HLC54; in the presence or absence of tyrosol, biofilms of strain JKC19 consisted of a mixture of yeasts and hyphae, whereas those of strains HLC52 and HLC54 contained yeast cells exclusively (results not shown). Thus, tyrosol was not able to stimulate hyphal formation in *C. albicans* strains defective in the Efg 1 signaling pathway.

Effect of tyrosol on early stages of biofilm formation. To investigate whether tyrosol accelerates the morphological conversion (yeast cells to filaments) during the early stages of biofilm formation, scanning electron microscopy was carried out at 1, 2, 3, and 6 h of incubation in the presence or absence of 50 μM tyrosol. Tyrosol was added at time zero of biofilm formation. The results (Fig. 8) show that tyrosol increases hyphal formation between 2 h and 6 h of biofilm development, relative to control biofilms incubated in the absence of tyrosol.

Effect of planktonic and biofilm culture supernatants on germ tube formation. To explore the biological impacts of tyrosol, farnesol, and other culture constituents produced during planktonic cell growth and biofilm formation, culture supernatants of *C. albicans* SC5314 were collected at 14, 24, 48, and 72 h and tested for their abilities to inhibit germ tube formation by the same *C. albicans* strain. This was accomplished by measuring cell dry weights at the times that supernatant samples were collected and by determining germ tube formation in the presence of the supernatants. The results showed that supernatants from planktonic cells after growth for 14, 24, 48, and 72 h inhibited germ tube formation by 1, 7, 17, and 20%, respectively (Fig. 9A). Supernatants from biofilms harvested at 24, 48, and 72 h also inhibited germ tube formation but to a greater extent (10, 28, and 42%, respectively) than those from planktonic cells (Fig. 9B). This suggests that farnesol activity exceeds that of tyrosol in all of these culture supernatants, resulting in an inhibition, not stimulation, of germ tube formation. A figure of 42% inhibition of germ tube formation equates to an active farnesol concentration of 13.5 μM as determined from a standard farnesol response curve (not shown). By contrast, supernatant from 14-h biofilms enhanced germ tube formation by 10%, indicating a higher activity for tyrosol at this stage in biofilm development (Fig. 9B).

When the percentage of inhibition or enhancement was calculated as a function of cell dry weight, it was again evident that biofilm cells produce significantly more tyrosol than do planktonic cells. The secretion of farnesol (and possibly other quorum-sensing molecules) also appeared to be greater in biofilms. For example, supernatant equivalent to 1 mg (dry weight) of biofilms formed at 24, 48, and 72 h inhibited germ tube formation by 11, 28, and 47%, respectively, whereas that from 1 mg (dry weight) of planktonic cells inhibited the process by only 5, 8, and 11%, respectively (Fig. 9C). On the other hand, 14-h supernatant from 1 mg (dry weight) of biofilm

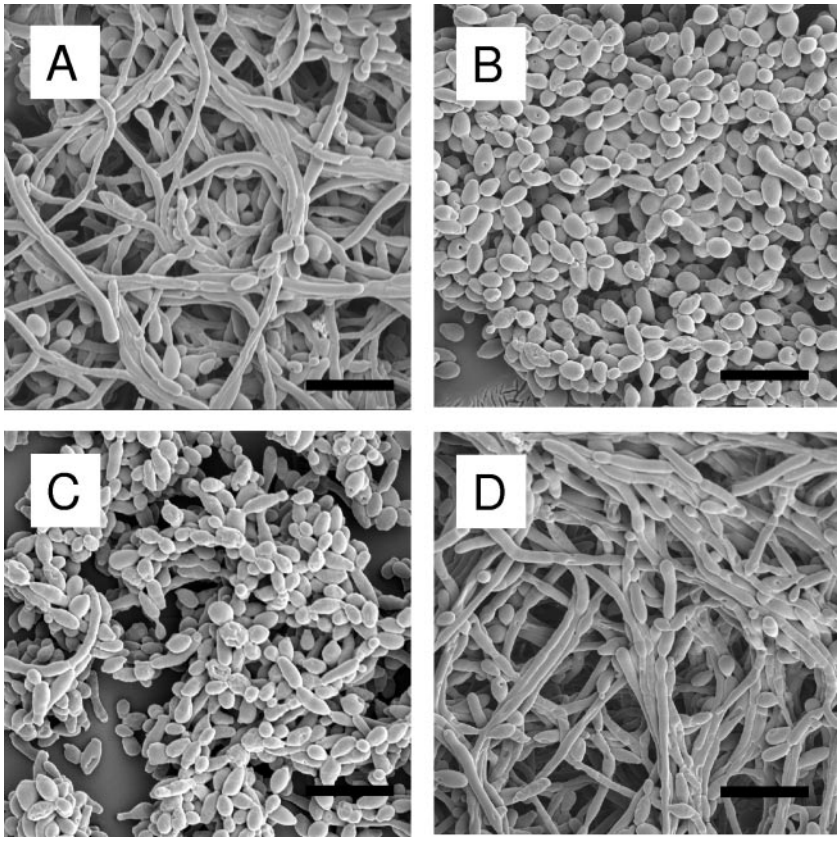

FIG. 7. Scanning electron micrographs of *C. albicans* SC5314 biofilms grown on polystyrene disks in the presence of farnesol and tyrosol. Biofilms were grown for 48 h in the presence of 1 mM farnesol and/or 500 μM tyrosol. (A) Control biofilm. (B) Farnesol-treated biofilm. (C) Farnesol-plus-tyrosol-treated biofilm. (D) Tyrosol-treated biofilm. Bar, 10 μm.

enhanced germ tube formation by 17%, while that from planktonic cells inhibited the morphological transition by 1% (Fig. 9C). These results suggest that the production of farnesol increases more during the later stages of biofilm formation than it does during planktonic cell growth. It also indicates that the activity of tyrosol exceeds that of farnesol in 14-h biofilms, but that after the incubation of planktonic cultures for the same time period, farnesol activity dominates slightly.

DISCUSSION

Tyrosol was identified in a recent study (5) as a quorum-sensing molecule that can play an important role in growth and morphogenesis in *C. albicans*. The supernatant recovered from high-density cultures contained tyrosol and this compound allowed highly diluted cultures to resume exponential growth without a substantial lag. The tyrosol activity was specific to the lag phase and had no effect on the exponential growth of *C. albicans.* Moreover, tyrosol accelerated the conversion of yeasts to filaments (5). The production of tyrosol by *Candida* species (7, 19) and *S. cerevisiae* (3, 26) has been noted in earlier reports. One of these studies (7) showed that tyrosol secretion by *C. albicans* protects the organism against phagocytic killing. Interestingly, significantly more tyrosol was secreted by *C. albicans* and *Candida tropicalis* than by *Candida glabrata* or *Candida parapsilosis*, suggesting a possible link with virulence (7). Tyrosol is one of the major phenolic compounds present in olive oil and is known to have potent antioxidant properties (17). However, a recent report (28) indicates that farnesol may be more important than tyrosol in protecting yeast cells from oxidative stress.

The results of this investigation demonstrate that tyrosol is produced by biofilms of *C. albicans* as well as by planktonic cells. Tyrosol was detected at a concentration of 8.7 μM in planktonic cultures of *C. albicans* SC5314 after 24 h of incubation; this is a higher concentration than that reported previously (approximately 3 μM) for the same strain (5). Increased tyrosol production could be due to the higher incubation temperature used here (37°C compared with 30°C used earlier). Alternatively, differences in the extraction and analysis protocols might explain the discrepancy; our procedure gives a demonstrably high recovery of tyrosol. However, our results show a clear correlation between tyrosol production and cell dry weight. These findings support the conclusions of Chen et al. (5) and further suggest that tyrosol, like farnesol, can act as a quorum-sensing molecule in biofilms as well as in planktonic cells. Moreover, mutants of *C. albicans* with defined defects in the Efg 1, the Cph 1, or both morphogenetic signaling pathways also produced tyrosol in a density-dependent fashion and at levels similar to that of the wild-type strain.

Biofilms secreted at least 50% more tyrosol than did planktonic cells for four different *C. albicans* strains, including the

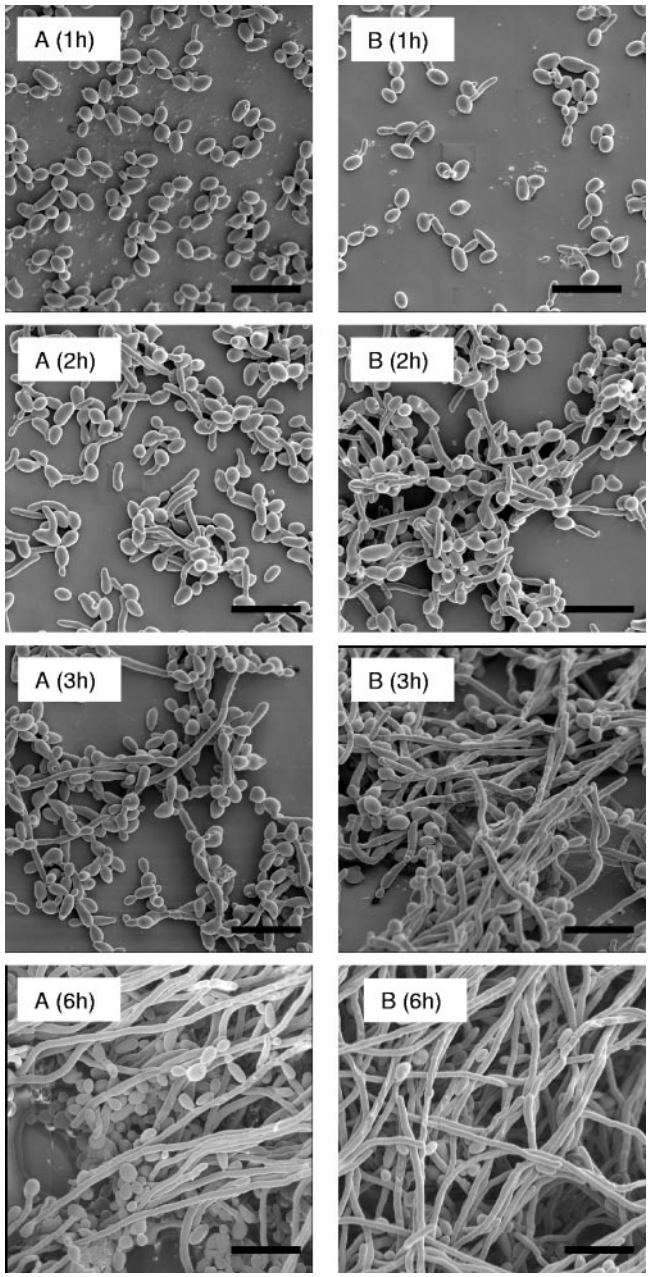

FIG. 8. Scanning electron micrographs showing the effect of tyrosol on the early stages of biofilm formation by *C. albicans* SC5314. Biofilms were grown on polyvinyl chloride catheter disks in the presence or absence of 50 μM tyrosol. (A) Control biofilms incubated for 1, 2, 3, and 6 h. (B) Tyrosol-treated biofilms incubated for 1, 2, 3, and 6 h. Bar, 10 μm.

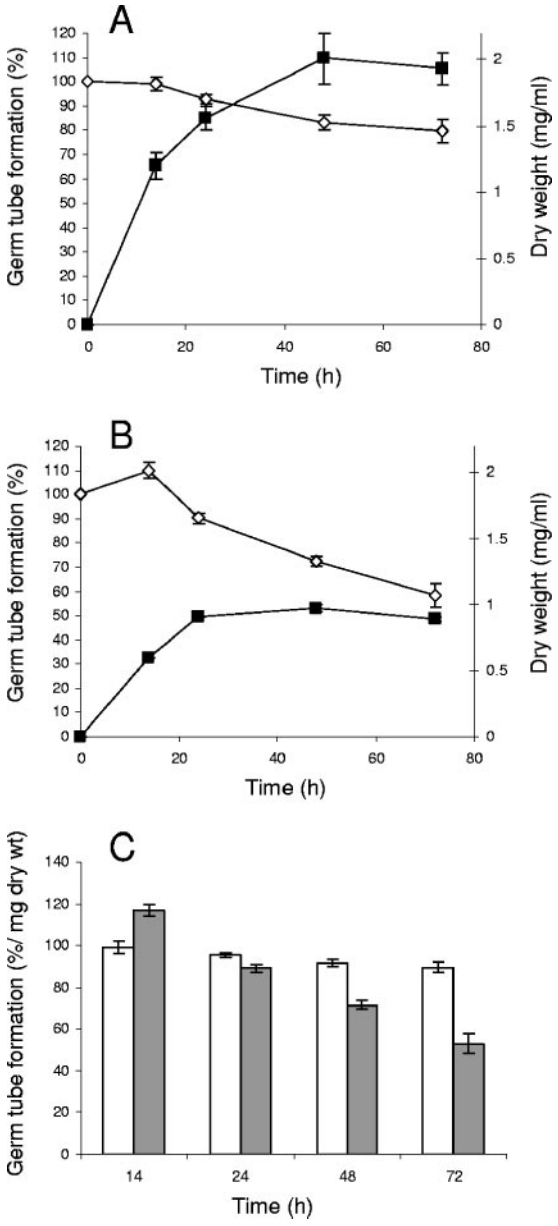

FIG. 9. Effects of planktonic and biofilm culture supernatants on germ tube formation by *C. albicans* SC5314. The inhibitory effect of culture supernatants on germ tube formation (◇) is shown, together with cell dry weight over 72 h (■) for planktonic cells (A) and biofilms (B). This percentage of inhibition of germ tube formation is also shown as a function of cell dry weight (C) for planktonic cultures (□) and biofilms (▨). Germ tube formation was determined as a percentage of that for control cells incubated in the absence of culture supernatants. Results are means ± SEMs of two independent experiments carried out in triplicate. Mean (±SEM) values for the controls ranged from 158 ± 7 to 180 ± 12 cells/200 cells counted.

mutants defective in signaling pathways. We recently reported a similarly enhanced production of prostaglandin by *C. albicans* biofilms (1). Purified fungal prostaglandin is known to have adverse effects on mammalian cells in vitro (21). It therefore seems likely that both tyrosol and prostaglandin could promote fungal pathogenesis if produced in high concentrations by biofilms in vivo. Like tyrosol, prostaglandins are small, relatively hydrophobic molecules which enhance germ tube formation (13, 21). The secretion of *C. albicans* prostaglandin, however, shows little correlation with cell density and there is currently no evidence to suggest that it functions as a quorum-sensing signal (1).

The effects of exogenous tyrosol and farnesol on biofilm formation were investigated by a quantitative catheter disk assay and by scanning electron microscopy. The addition of farnesol at a very early stage inhibited biofilm formation when

assessed after incubation for 48 h. This finding essentially confirms that of Ramage et al. (25), who used a multiwell plate biofilm system and reported an inhibition of around 85% with 300 μM farnesol. However, the extent of inhibition observed here was substantially lower, even at higher concentrations of farnesol. It is now known that the concentration of farnesol required to inhibit germ tube formation by planktonic cells is considerably higher (up to 250 μM) in serum than in defined medium (1 μM) due to binding of the lipophilic farnesol to serum albumins (18). The high farnesol concentrations needed for the inhibition of biofilm formation may similarly be due to farnesol adsorption to the biofilm substratum (here, polyvinyl chloride or polystyrene), which would effectively lower the concentration of farnesol in solution. The requirement that farnesol be added early in the process is not surprising since it has been shown that, while farnesol blocks the yeast-mycelium conversion in *C. albicans*, it does not prevent the elongation of preexisting hyphae (18, 20). There is therefore a limited time in which cells can respond to farnesol (20). As noted above, growth medium composition can also affect farnesol activity (18). In the first report on *Candida* biofilms from this laboratory (10), we observed that YNB-glucose, the medium used here, produces only yeast cells in liquid culture or on agar but a mixture of yeasts and hyphae in adherent populations on catheter disks, suggesting a form of contact-induced gene expression in biofilms. Such a pathway has now been identified in *C. albicans* (14). Physical contact with a surface results in the activation of the mitogen-activated protein kinase Mkc1p which is part of the fungal cell integrity pathway; *mkc1*-null mutants produce abnormal biofilms with reduced filamentation (14). Hypha production triggered via surface contact, rather than by a component of the growth medium, might be less susceptible to inhibition by farnesol. This, in turn, could account for the different levels of inhibition observed here and in the earlier investigation (25), in which a hypha-inducing medium was used.

In contrast with the farnesol addition, the addition of tyrosol at an early stage had no significant effect on biofilm formation as measured by the XTT assay after 48 h. Mature (48-h) biofilms grown in the presence or absence of tyrosol also had similar morphologies, as determined by scanning electron microscopy. However, tyrosol appeared to accelerate the production of hyphae between 2 and 6 h of biofilm development. It is possible that, like farnesol, exogenous tyrosol affects only the early stages of biofilm development, before some cells are already committed to hyphal growth. An alternative explanation might be that minimal endogenous tyrosol is produced during this period (up to 0.1 μM at 5 h). Between 10 and 24 h, there is a rapid increase in tyrosol concentration and then a gradual leveling off for most strains. Presumably during these intermediate stages, endogenous tyrosol production would reach a threshold concentration sufficient to promote yeast-hypha morphogenesis.

To investigate the relative importance of tyrosol and farnesol on biofilm formation, the two quorum-sensing molecules were added simultaneously early in the process. The effects of relatively low concentrations of farnesol (50 μM) could be abolished by higher concentrations of tyrosol. However, the inhibition of biofilm formation by 1 mM farnesol was unaffected by tyrosol at any concentration tested, including an equimolar concentration. Moreover, biofilms formed in the presence of both farnesol and tyrosol consisted almost entirely of yeasts, supporting the conclusion that farnesol has a dominant effect on cell morphology. Recently, Cao et al. (4) used cDNA microarray analysis to investigate gene expression in *C. albicans* biofilms grown in the presence or absence of farnesol. A total of 274 genes were identified as responsive in farnesol-treated biofilms, with 104 genes up-regulated and 170 genes down-regulated. Several genes involved in hypha formation were differentially expressed; the *TUP1* gene was up-regulated, while the *PDE2* and *CRK1* genes were down-regulated. The cell surface hydrophobicity-associated gene *CSH1* was also down-regulated, and some genes related to drug resistance were differentially expressed (4). Analogous studies of differential gene expression in tyrosol-treated biofilms have not been reported.

In vivo, the physiological roles of tyrosol and farnesol are likely to depend on their respective concentrations at a particular stage during biofilm formation and on the concentration of each quorum-sensing molecule required to trigger or suppress gene expression. To investigate the relative concentrations of these compounds produced at different stages of biofilm formation, biofilm supernatants were tested for their abilities to inhibit or enhance germ tube formation by planktonic cells. The results suggested that tyrosol activity exceeds that of farnesol after 14 h but that this is reversed after 24 h. Moreover, farnesol production appears to increase significantly during the later stages (48 to 72 h) of biofilm formation. These are preliminary findings and accurate, simultaneous, chemical determinations of farnesol and tyrosol are clearly required. However, our results suggest that tyrosol promotes hyphal formation during the early and intermediate stages of biofilm development. Farnesol, on the other hand, may play a critical role in the later stages by stimulating the release of yeast cells from the biofilm and so permitting the dispersal of the organism to colonize new surfaces. This has already been proposed as an important function of quorum sensing in bacteria (23). It is likely that other quorum-sensing molecules also participate in biofilm development. Two further aromatic alcohols, phenylethanol and tryptophol, are produced by *C. albicans* in addition to tyrosol (6, 15). Recently, they were reported to inhibit both germ tube formation and biofilm development at high concentrations (6), but their physiological roles are not yet clear.

ACKNOWLEDGMENTS

Mohammed Alem and Mohammed Oteef are the recipients of research studentships from the Ministry of Health, Saudi Arabia, and King Khalid University, Saudi Arabia, respectively.

We are indebted to Margaret Mullin for expert assistance with electron microscopy.

REFERENCES

1. **Alem, M. A. S., and L. J. Douglas.** 2005. Prostaglandin production during growth of *Candida albicans* biofilms. J. Med. Microbiol. **54:**1001–1005.
2. **Baillie, G. S., and L. J. Douglas.** 1999. *Candida* biofilms and their susceptibility to antifungal agents. Methods Enzymol. **310:**644–656.
3. **Batrakov, S. G., G. I. El'Registan, N. N. Pridachina, V. A. Nenashev, A. N. Kozlova, M. N. Griaznova, and I. N. Zolotareva.** 1993. Tyrosol—the autoregulatory d1 factor of the yeast *Saccharomyces cerevisiae.* Mikrobiologiya **62:**633–638.
4. **Cao, Y.-Y., Y.-B. Cao, Z. Xu, K. Ying, Y. Li, Y. Xie, Z.-Y. Zhu, W.-S. Chen, and Y.-Y. Jiang.** 2005. cDNA microarray analysis of differential gene expres-

sion in *Candida albicans* biofilm exposed to farnesol. Antimicrob. Agents Chemother. **49:**584–589.
5. **Chen, H., M. Fujita, Q. Feng, J. Clardy, and G. R. Fink.** 2004. Tyrosol is a quorum sensing molecule in *Candida albicans.* Proc. Natl. Acad. Sci. USA **101:**5048–5052.
6. **Chen, H., and G. R. Fink.** 2006. Feedback control of morphogenesis in fungi by aromatic alcohols. Genes Dev. **20:**1150–1161.
7. **Cremer, J., V. Vatou, and I. Braveny.** 1999. 2,4-(Hydroxyphenyl)-ethanol, an antioxidative agent produced by *Candida* spp., impairs neutrophilic yeast killing in vitro. FEMS Microbiol. Lett. **170:**319–325.
8. **Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg.** 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science **280:**295–298.
9. **Douglas, L. J.** 2003. *Candida* biofilms and their role in infection. Trends Microbiol. **11:**30–36.
10. **Hawser, S. P., and L. J. Douglas.** 1994. Biofilm formation by *Candida* species on the surface of catheter materials in vitro. Infect. Immun. **62:**915–921.
11. **Hornby, J. M., E. C. Jensen, A. D. Lisec, J. J. Tasto, B. Jahnke, R. Shoemaker, P. Dussault, and K. W. Nickerson.** 2001. Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. Appl. Environ. Microbiol. **67:**2982–2992.
12. **Huber, B., K. Riedel, M. Hentzer, A. Heydorn, A. Gotschlich, M. Givskov, S. Molin, and L. Eberl.** 2001. The *cep* quorum-sensing system of *Burkholderia cepacia* H111 controls biofilm formation and swarming motility. Microbiology **147:**2517–2528.
13. **Kalo-Klein, A., and S. S. Witkin.** 1990. Prostaglandin E2 enhances and gamma interferon inhibits germ tube formation in *Candida albicans.* Infect. Immun. **58:**260–262.
14. **Kumamoto, C. A.** 2005. A contact-activated kinase signals *Candida albicans* invasive growth and biofilm development. Proc. Natl. Acad. Sci. USA **102:**5576–5581.
15. **Lingappa, B. T., M. Prasad, Y. Lingappa, D. F. Hunt, and K. Biemann.** 1969. Phenethyl alcohol and tryptophol: autoantibiotics produced by the fungus *Candida albicans.* Science **163:**192–194.
16. **Lo, H.-J., J. R. Kohler, B. DiDomenico, D. Loebenberg, A. Cacciapuoti, and G. R. Fink.** 1997. Nonfilamentous *C. albicans* mutants are avirulent. Cell **90:**939–949.
17. **Moreno, J. J.** 2003. Effect of olive oil minor components on oxidative stress and arachidonic acid mobilization and metabolism by macrophages RAW 264.7. Free Radic. Biol. Med. **35:**1073–1081.
18. **Mosel, D. D., R. Dumitru, J. M. Hornby, A. L. Atkin, and K. W. Nickerson.** 2005. Farnesol concentrations required to block germ tube formation in *Candida albicans* in the presence and absence of serum. Appl. Environ. Microbiol. **71:**4938–4940.
19. **Narayanan, T. K., and G. R. Rao.** 1976. Production of beta-(4-hydroxyphenyl)ethanol and beta-(4-hydroxyphenyl)lactic acid by *Candida* species. Can. J. Microbiol. **22:**384–389.
20. **Nickerson, K. W., A. L. Atkin, and J. M. Hornby.** 2006. Quorum sensing in dimorphic fungi: farnesol and beyond. Appl. Environ. Microbiol. **72:**3805–3813.
21. **Noverr, M. C., S. M. Phare, G. B. Toews, M. J. Coffey, and G. B. Huffnagle.** 2001. Pathogenic yeasts *Cryptococcus neoformans* and *Candida albicans* produce immunomodulatory prostaglandins. Infect. Immun. **69:**2957–2963.
22. **Oh, K.-B., H. Miyazawa, T. Naito, and H. Matsuoka.** 2001. Purification and characterization of an autoregulatory substance capable of regulating the morphological transition in *Candida albicans.* Proc. Natl. Acad. Sci. USA **98:**4664–4668.
23. **Parsek, M. R., and E. P. Greenberg.** 2005. Sociomicrobiology: the connections between quorum sensing and biofilms. Trends Microbiol. **13:**27–33.
24. **Puskas, A., E. P. Greenberg, S. Kaplan, and A. L. Schaefer.** 1997. A quorum-sensing system in the free-living photosynthetic bacterium *Rhodobacter sphaeroides*. J. Bacteriol. **179:**7530–7537.
25. **Ramage, G., S. P. Saville, B. L. Wickes, and J. L. Lopez-Ribot.** 2002. Inhibition of *Candida albicans* biofilm formation by farnesol, a quorum-sensing molecule. Appl. Environ. Microbiol. **68:**5459–5463.
26. **Sentheshanmuganathan, S., and S. R. Elsden.** 1958. The mechanism of the formation of tyrosol by *Saccharomyces cerevisiae.* Biochem. J. **69:**210–218.
27. **Swift, S., J. A. Downie, N. A. Whitehead, A. M. L. Barnard, G. P. C. Salmond, and P. Williams.** 2001. Quorum sensing as a population-density-dependent determinant of bacterial physiology. Adv. Microb. Physiol. **45:**199–270.
28. **Westwater, C., E. Balish, and D. A. Schofield.** 2005. *Candida albicans*-conditioned medium protects yeast cells from oxidative stress: a possible link between quorum sensing and oxidative stress resistance. Eukaryot. Cell **4:**1654–1661.