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1 **High level biosynthesis of the mycosubtilin antibiotic anteiso-C17 isoform in *Bacillus***
2 ***subtilis* and characterisation of its candidacidal activity.**

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26 **Abstract**

27 High-level production (880 mg l⁻¹) and isolation of the anteiso-C17 isoform of the lipopeptide
28 mycosubtilin, produced by a genetically engineered *B. subtilis* strain is reported. Antifungal
29 activity of this isoform, as determined via culture, fluorometric and cell leakage assays,
30 suggest its potential therapeutic use as antifungal agent, in particular on *Candida* sp.

31

32

33 **Short-form paper**

34 The soil bacterium *Bacillus subtilis* ATCC 6633 synthesises the lipopeptides
35 mycosubtilin and surfactin by so-called non-ribosomal peptide synthetase. Mycosubtilin
36 belongs to the iturin family and is composed of seven α -amino acids linked to a unique C16 or
37 C17 β -amino fatty acid with a linear or branched (iso or anteiso) acyl chain (15). This
38 amphiphilic structure confers interesting biological properties to this secondary metabolite,
39 particularly antifungal activity, which increases with the number of carbon atoms of the acyl
40 chain (10). However, studies and applications of mycosubtilin are compromised by limited
41 production by the native producer, co-synthesis of surfactin, and the existence of different
42 mycosubtilin homologues and isoforms. In this work, utilisation of specific precursors
43 together with appropriate culture conditions for a genetically engineered strain led to the
44 synthesis of a large amount of the most biologically active mycosubtilin homologue.
45 Structural characterisations by mass spectrometry and NMR spectroscopy demonstrated that
46 this homologue corresponds to the anteiso-C17 isoform. Its antifungal activity on pathogenic
47 *Candida* sp. was examined by MIC determination, fluorescence spectroscopy and leakage
48 experiments.

49 Overproduction of mycosubtilin in *B. subtilis* ATCC 6633 was obtained by
50 replacement of the native promoter of the *myc* operon which encodes mycosubtilin synthetase
51 by the strong and tightly regulated *xylA* promoter from the *B. megaterium* xylose isomerase
52 (16). To this end, a repressor-promoter *xylR-pxylA* fragment was PCR-amplified from pAXO1
53 (9) with *pfu* DNA polymerase (Promega) and primers R100/R101 before being cloned at the
54 *HincII* site of pBG103 to yield pBG113 (Tables 1 and 2). Then, a spectinomycin resistance
55 cassette was rescued from pRFB122 by *PstI/EagI* digestion and inserted into pBG113 at the
56 corresponding site to yield pBG113s (Table 1). This latter construct was used to transform *B.*
57 *subtilis* ATCC 6633 as previously described (8) and transformants were selected on Luria-

58 Bertani plates supplemented with spectinomycin (100 $\mu\text{g/ml}$). Correct integration in the
59 resulting RFB107 strain was verified by analytical PCR using primers R102/R103 (Table 2).
60 In a second step, the *srf* operon encoding surfactin synthetase was disrupted in RFB107 to
61 render the strain unable to synthesise surfactin. The knockout was targeted downstream of the
62 *comS* regulator involved in competence mechanisms which lies nested and out of frame
63 within the *srf* operon (5, 6). The disruption cassette was obtained by a ligation-mediated PCR
64 method (12) as follows: fragments of *srfAB* and *srfAD* ORFs were PCR-amplified (primers
65 R106/R107 and R108/R109, respectively) from *B. subtilis* ATCC 6633 genomic DNA
66 (obtained with the Wizard Genomic DNA purification kit, Promega) while the erythromycin
67 resistance cassette (ERY) of pDML1567 was amplified with primer R110/R111. PCR
68 fragments (200 ng) were *Sfi*I-digested, purified with the GFX purification kit (GE Healthcare)
69 and ligated with T4 DNA ligase (Promega) before being used as a template for PCR
70 amplification using primer R106/R109. The resulting *srfAB*-ERY-*srfAD* cassette was then
71 used to transform *B. subtilis* RFB107 and transformants were selected on LB erythromycin
72 plates (1 $\mu\text{g/ml}$). Integration by double crossing event in the resulting strain RFB112 was
73 verified by analytical PCR using primer R104/R105 (Table I) and by LC-ESI-MS analysis of
74 the purified culture supernatant as described elsewhere (14).

75 For mycosubtilin production, strain RFB112 was cultured at 25°C for 48 h in a
76 medium containing yeast extract 1 g l⁻¹, sucrose 15 mM, xylose 75 mM, isoleucine 15 mM,
77 K₂HPO₄ 10 mM, MgSO₄ 4 mM and KCl 6 mM. Mycosubtilin concentration in the culture
78 broth, determined by reverse-phase HPLC on a C18 X-Terra column (4.6 x 15 mm, 3.5 μmX ,
79 Waters) (8), was 880 mg l⁻¹. This represents a 50-fold increased production yield compared to
80 the native strain for a culture time reduced by up to 40% (8). For mycosubtilin purification,
81 the culture supernatant was loaded onto a 50 ml C18 ODS matrix (Macherey-Nagel)
82 equilibrated with 10 bed volumes of MilliQ water. The matrix was subsequently washed with

83 MilliQ water and with a mixture of MilliQ water:methanol (1:1 v/v) (5 bed volumes each).
84 Crude mycosubtilin was then eluted with 3 bed volumes of methanol before being 10-fold
85 concentrated by rotary evaporation. Further purification was performed by reverse-phase
86 HPLC and the peak corresponding to the mycosubtilin was collected and vacuum-dried. The
87 purified molecule, analyzed by MALDI-TOF mass spectrometry as previously described (13),
88 showed signals at m/z 1107.5 and 1123.5 Da which are in accordance with the calculated
89 mass values of Na^+ and K^+ adducts of the C17 mycosubtilin homologue. Additional ions
90 corresponding to C16 homologues were not detected, suggesting that the latter were not
91 synthesized under these conditions (Fig. 1). Acyl chain isomery was further characterised
92 either by ^1H and ^1H - ^{13}C HSQC NMR spectroscopy (18) on a Bruker 500 MHz spectrometer at
93 25°C with a 10 mM solution of purified mycosubtilin in methanol- d_4 . In the high-field methyl
94 region, two signals were observed at (δ_{H} 0.89, δ_{C} 18.5) and (δ_{H} 0.93, δ_{C} 13), corresponding to
95 a doublet and a triplet with J values of 6.9 Hz and 7.4 Hz, respectively (Fig. 2 and not shown).
96 These signals with very low proton chemical shift (δ_{H} 0.89 and δ_{H} 0.93) correspond to
97 methyl groups of the long chain β -amino acid found in iturin lipopeptide (11). The proton
98 multiplicity (i.e. doublet and triplet) observed for this two methyl groups could only account
99 for the anteiso isomery of the heptadecanoic acyl chain of mycosubtilin.

100 The biological activity of the purified anteiso-C17 mycosubtilin was first characterised
101 by determining the susceptibility of an array of yeasts and molds. As shown in Table 3, a
102 strong antifungal activity was obtained for yeast strains whereas no significant growth
103 inhibition was observed for molds. Despite varying susceptibilities toward both yeast species
104 and specific isolates of the same species, the relatively low MIC values obtained, especially
105 for isolates resistant to conventional drugs (fluconazole and/or amphotericin B), point to the
106 powerful properties of this particular mycosubtilin isoform. The moderate effectiveness
107 against the sterol auxotrophic *C. glabrata* isolates could be explained by the presence of free

108 ergosterol in the culture medium which is known as a strong iturin antagonist (2). For iturin
109 A, a closely related iturinic lipopeptide to mycosubtilin, a membrane pore-forming mode of
110 action has been suggested for *Saccharomyces cerevisiae* spheroplast (3). Accordingly, the
111 antifungal activity of the purified mycosubtilin was further investigated by determining its
112 ability to induce cell membrane destabilisation by measuring the transmembrane electrical
113 potential ($\Delta\Psi$) with the fluorescent probe 3'-dipropylthia dicarbocyanine iodide [DiSC₃ (5)]
114 (7). As shown in Fig. 3, the significant fluorescence signal observed upon addition of 10 μ M
115 of mycosubtilin to a *C. albicans* reference strain ATCC 10231 cell suspension loaded with
116 fluorescent dye demonstrated the complete disruption of the $\Delta\Psi$ (as shown by the lack of an
117 additional fluorescence signal upon addition of the ionophore valinomycin). At the same time,
118 an extensive cell leakage of UV-absorbing molecules (i.e. protein and nucleic acids; for
119 details see ref. (17)) could be observed, suggesting the formation of transmembrane pores due
120 to the action of mycosubtilin (not shown).

121 Considering that the antifungal activity of iturinic lipopeptides increases with the length
122 of their fatty acid moiety and that mycosubtilin is considered as the most active iturin (10),
123 the aim of this study was to specifically overproduce a C17 mycosubtilin homologue. As this
124 lipopeptide is of great fundamental interest and shows great antibiotic potential, its study is
125 particularly worthwhile. The extent to which mycosubtilin overproduction occurs in the
126 engineered strain is greater than what has ever been observed to date (13). In combination
127 with low culture temperature, which also contributes to this high level of production (8),
128 addition of isoleucine made it possible to direct mycosubtilin synthesis toward the anteiso-
129 C17 isoform with high efficiency. This preliminary investigation on the antifungal properties
130 of this particular mycosubtilin isoform is very promising and must be pursued. We are
131 currently investigating the mechanism of antifungal activity in greater detail, especially on
132 *Candida* strains less susceptible to conventional antifungal drugs.

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134

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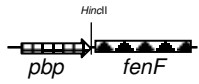

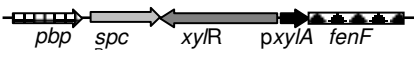
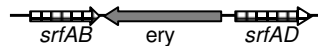
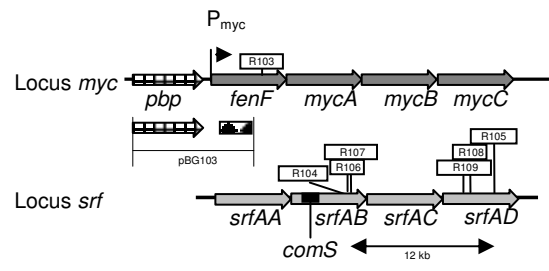
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TABLE 1. Plasmids and *Bacillus* strains used in this study

Plasmids	Description or genotype	Source or reference
pBG103		(13)
pBG113		This work
pBG113s		This work
pRFP129		This work
pRFB122	pGEMTeasy carrying a spectinomycin resistance gene	Fickers, unpublished
PDML1567	pGEMTeasy carrying a erythromycin resistance gene	Fickers, unpublished
<i>B. subtilis</i>		
ATCC6633	Wild-type strain producing mycosubtilin and surfactin	Lab stock
		
RFB107	ATCC 6633 overproducing derivative, xylose inducible	This work
RFB112	RFB107 derivative surfactin non-producer	This work

204

TABLE 2. Synthetic primers used for PCR amplification

Name	Sequence (5'-3')	Restriction site
R100	GGGAGCTCGGATCCCATTTC	-
R101	CGATATCTCTGCAGTCGCGATG	-
R102	CCACTCCTTTGTTTATCCACCGAAC	-
R103	GACGTTCAAATAAGTGTGATTGGCC	-
R104	ACGGAGGGAGACGATTTGCA	-
R105	ACATTCGGTGAATAAGGAAGCA	-
R106	GTGAAAATCCGAGGCTACCGC	-
R107	GGGGGCCCCAGCGGCCATATAAGCCGCCAGCTGGCG	<i>Sfi</i> I
R108	CAGGGGCCAGTGGGCCGCAGGGCGAAACGCTAGATAGG	<i>Sfi</i> I
R109	GCTGTCACAAACGGAAGAAGTC	
R110	CATGGCCCACTGGGCCCTGCTTCCTAATGCAGGAGTCGC	<i>Sfi</i> I
R111	CCCCGGCCGCTGGGGCCCCCGGATCGCCTATTTGGC	<i>Sfi</i> I

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TABLE 3 : *In vitro* susceptibility of different yeasts and molds toward purified anteiso-C17 mycosubtilin. Results are mean values of four independent experiments. Breakpoints for the determination of antibiotic resistance were determined according to the M27-A3 procedure (4). Experiments were performed in triplicate.

Strain	MIC ($\mu\text{g/ml}$)	Origin or Reference
<i>Sacharomyces cerevisiae</i>	4	Lab stock
<i>Yarrowia lipolytica</i> CBS6303	8	CBS
<i>Pichia pastoris</i>	32	Lab stock
<i>Candida albicans</i> ATCC10231 ^A	32	ATCC
<i>Candida albicans</i> IHEM3742	64	IHEM
<i>Candida. albicans</i> ^A	18	Lab stock
<i>Candida parapsilosis</i> IHEM9557	128	IHEM
<i>Candida tropicalis</i> IHEM6246	16	IHEM
<i>Candida tropicalis</i> ^{A, B, C}	16	Lab stock
<i>Candida guilliermondii</i> IHEM1067	128	IHEM
<i>Candida glabrata</i> IHEM6161	16	IHEM
<i>Candida glabrata</i> L999	2	(1)
<i>Candida glabrata</i> S53452 ^{A, C, D}	150	(1)
<i>Candida glabrata</i> H34736 ^{A, C, D}	150	(1)
<i>Candida glabrata</i> W16119 ^{A, C, D}	150	(1)
<i>Candida glabrata</i> ^{A, C}	16	Lab stock
<i>Cryptococcus neoformans</i> IHEM3969	8	IHEM
<i>Aspergillus parasiticus</i> IHEM4383	> 300	IHEM
<i>Aspergillus terreus</i> IHEM2499	> 300	IHEM
<i>Aspergillus fumigatus</i> IHEM3562	> 300	IHEM

IHEM : Biomedical fungi and yeasts collection (<http://bccm.belspo.be>); ATCC : American type culture collection (<http://www.lgcpromochem-atcc.com>), CBS : Centraalbureau voor Schimmelcultures, Fungal and Yeast Collection (<http://www.cbs.knaw.nl/databases/index.htm>)

^A resistant to fluconazole

^B resistant to amphotericin B

^C clinical isolate

^D auxothroph for sterol, culture medium was supplemented with ergosterol 20 $\mu\text{g/ml}$

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212 **Figure legends**

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214 **Figure 1** : Matrix-assisted laser desorption/ionization-time of flight spectra of lipopeptide
215 produced by *B. subtilis* RFB112 .

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217 **Figure 2** : Detail of ^1H - ^{13}C HSQC spectra of lipopeptide produced by *B. subtilis* strain
218 RFB112. Spectra were acquired according to (18). The methyl signals are indicated by an
219 arrow.

220

221 **Figure 3** : Transmembrane electrical potential ($\Delta\Psi$) determination of a *C. albicans* ATCC
222 10231 suspension (0.5 McFarland). Fluorophore Disc(3)5 (1 μM , arrow 1), mycosubtilin (15
223 μM , arrow 2) and valinomycin (1 μM , arrow 3) were added to the cell suspension after 50,
224 600 and 1000 seconds, respectively. Displayed data are one representative result of three
225 independent experiments.

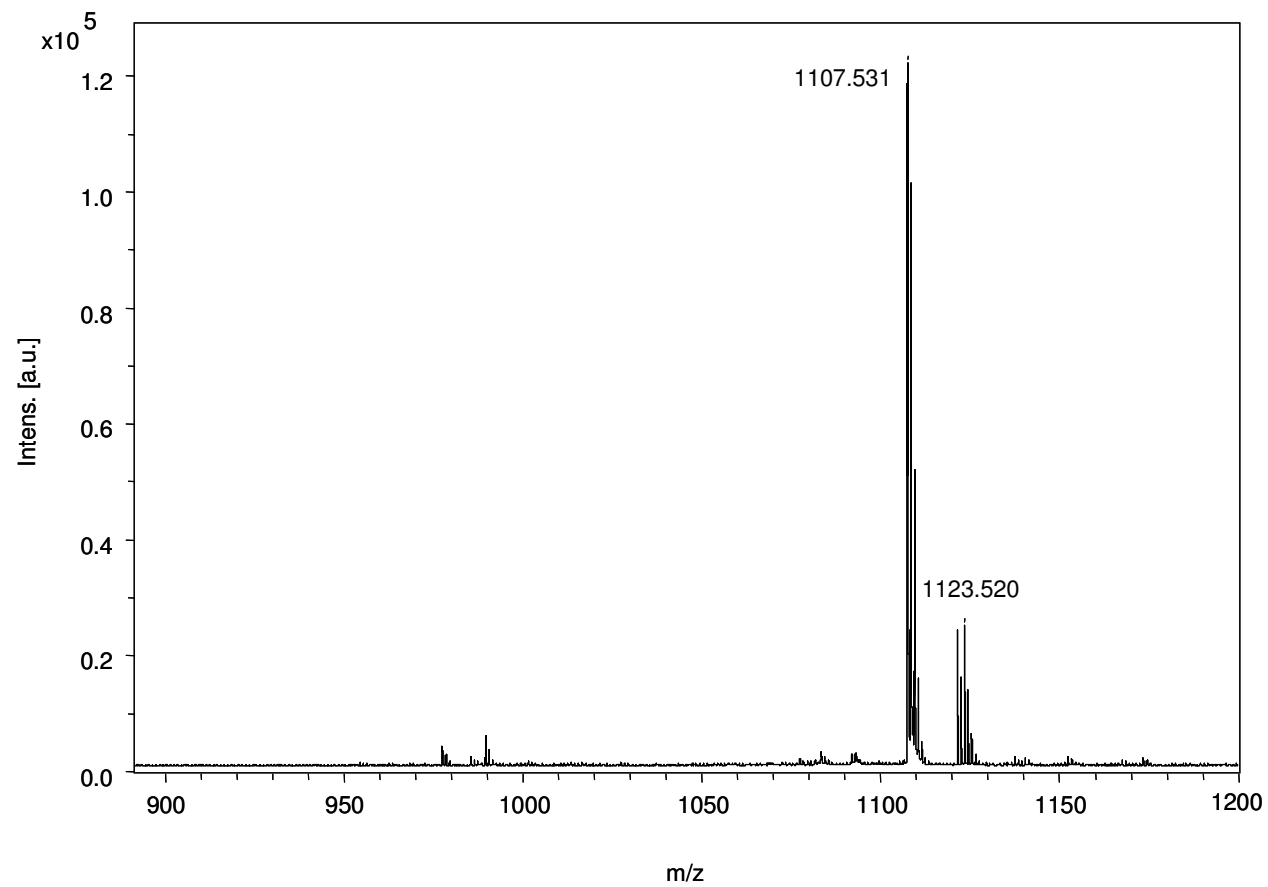
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227 **Figure 1 : MALDI-TOF spectra of lipopeptide produced by *B. subtilis* strain RFB112**

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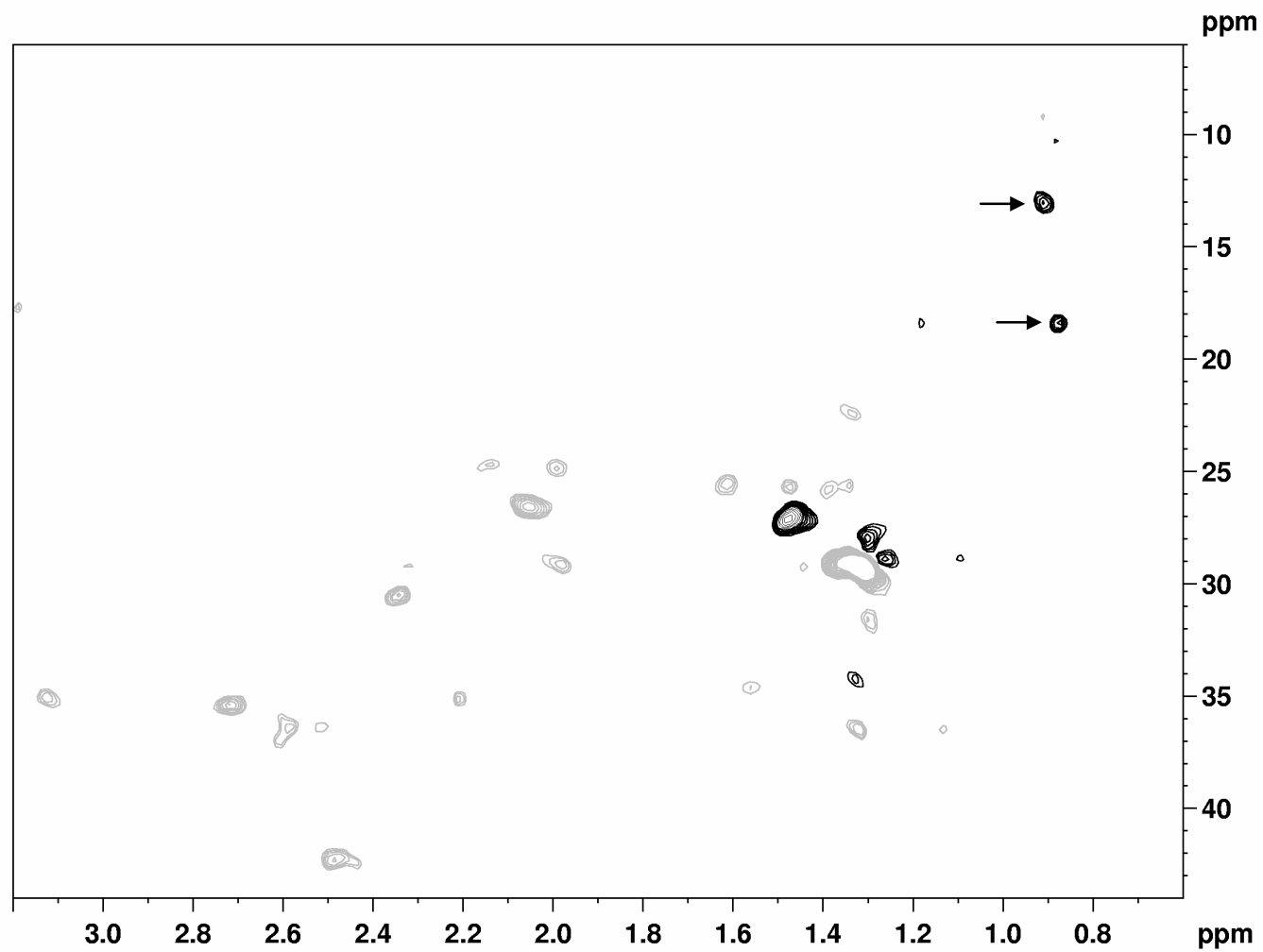


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233 **Figure 2 : Detail of ^1H - ^{13}C HSQC spectra of lipopeptide produced by *B. subtilis* strain RFB112**

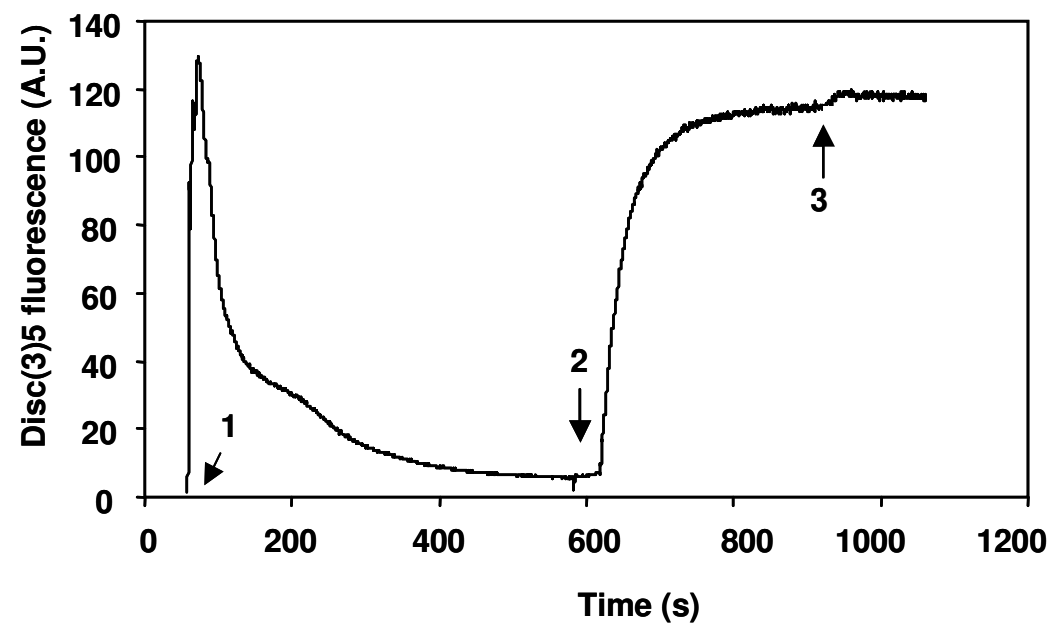
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240 **Figure 3 : Transmembrane electrical potential ($\Delta\Psi$) determination of a *C. albicans* ATCC 10231 suspension**

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